Impact of long-acting contraceptives on female genital tract cytokine profiles in a randomised controlled trial

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Contents

List of abbreviations 5
List of figures 7
List of tables 7
Summary 8
Background 9
Introduction 9
Immunity in the Female Genital Tract 9
Inflammation and HIV Risk 10
The Role of Bacterial Vaginosis and Sexually Transmitted Infections in Genital Inflammation and HIV Risk 12
The Role of the Hormonal Cycle in Inflammation and HIV Risk 13
Contraceptives and HIV risk 15
Injectables 15
Intrauterine devices 17
Implants 17
Mechanisms underlying the possible relationship between contraceptives and HIV risk 17
Impact of long-acting contraceptives on FGT immunity 17
Impact of contraceptives on cervicovaginal structure 19
Impact of synthetic hormones on endogenous hormones 20
HCs and the acquisition of STIs and BV 20
Rationale 22
Aims 22
Methods and Materials 23
Study participants 23
Specimen collection 23
STI diagnosis 23
Lateral vaginal wall swab processing and aliquoting 23
Cytokine, antimicrobial peptide and prostate specific antigen measurement 24
Data analysis 24
Results 25
Baseline Demographics 25
Immune mediator detection 32
Analysis of absolute immune mediator concentrations 33
Analysis of fold change in immune mediator concentrations 39
Principal component analysis 43
Unsupervised hierarchical clustering 49
Longitudinal changes in immune mediators 51
Changes in cytokine concentrations after adjustment for potential confounders 54
Discussion 57
Quality of mediator data 57

Baseline correlates of FGT immune mediator profiles 57
Changes in FGT immune mediator concentrations associated with contraceptive use 60
Copper IUD 60
DMPA-IM 63
LNG implant 64
Limitations 66
Conclusion 66
References 68
Appendix 76
Luminex assay principle 76
Standard curves 77
Line graphs of genital immune mediator concentrations over time 78
Log_{10}-transformed fold change analysis of immune mediator data 81
Baseline immune mediator profiles of women positive for *Chlamydia trachomatis* 83
Average age of women from SRU and MRU 84

List of abbreviations

BV – Bacterial vaginosis

CCR – C-C Chemokine receptor

COC – Combined oral contraceptive

Cu-IUD – copper intrauterine device

DMPA – Depomedroxyprogesterone acetate

DMPA-IM – intramuscular DMPA

ECHO – Evidence for Contraceptive Options and HIV Outcomes

FC – fold change

FGT – Female genital tract

GR – Glucocorticoid receptor

HC – Hormonal contraceptive

HIV – Human Immunodeficiency Virus

HSV-2 – Herpes Simplex Virus 2

HPV – Human Papillomavirus

Ig – Immunoglobulin

IL - Interleukin

IP – Interferon-gamma induced protein

IUD – Intrauterine device

LNG – Levonorgestrel

MCP – Monocyte chemoattractant protein

MIP – Macrophage inflammatory protein

MOC – magnitude of change

MR – Mineralocorticoid receptor

NET-EN - Norethisterone enanthate

NF-kB – Nuclear Factor kB

PCA – Principal component analysis

PR – Progesterone receptor

PSA – Prostate specific antigen

RANTES - Regulated on activation, normal T cell expressed and secreted

RNA – Ribonucleic acid

SLPI – secretory leukocyte protease inhibitor

SSA – Sub-Saharan Africa

STI – Sexually transmitted infection

TNF – Tumour necrosis factor

List of figures

Figure 1. Sexually transmitted infection (STI) prevalence (%) across sites……29

Figure 2. Prostate specific antigen (PSA) concentrations across sites……..29

Figure 3: Correlation between duplicate immune mediator concentrations……..….30

Figure 4: % Undetected immune mediator concentrations……...31

Figure 5: Genital immune mediator concentrations (pg/mL) in copper IUD users……33

Figure 6: Genital immune mediator concentrations (pg/mL) in DMPA-IM users……..34

Figure 7: Genital immune mediator concentrations (pg/mL) in LNG implant users……35

Figure 8: 36

Figure 9: Fold change of genital immune mediator concentrations one month following initiation of contraceptive method……39

Figure 10: Fold change of genital immune mediator concentrations three months following initiation of contraceptive method……40

Figure 11: Cumulative median fold change values of immune mediators following contraceptive initiation……41

Figure 12: Magnitude of change in principal component estimates for pro-inflammatory cytokine profiles, chemokine profiles and overall immune mediator profiles one month after contraceptive initiation……43

Figure 13: Magnitude of change in principal component estimates for pro-inflammatory cytokine profiles, chemokine profiles and overall immune mediator profiles three months after contraceptive initiation……43

Figure 14: Principal component analysis of log-transformed fold change in immune mediator concentrations following contraceptive initiation……...45

Figure 15: Unsupervised hierarchical clustering of immune mediator profiles following contraceptive initiation……..48

List of tables

Table 1: Baseline Demographic and clinical characteristics by immune mediator……25

Table 2: Correlation analysis of duplicate concentrations per immune mediator……..31

Table 3: Mixed effects linear regression analysis of changes in immune mediator concentrations in copper IUD relative to DMPA-IM users……50

Table 4: Mixed effects linear regression analysis of changes in immune mediator concentrations in LNG implant relative to DMPA-IM users……51

Table 5: Multinomial logistic regression analysis of changes in immune mediator concentrations in copper IUD compared to DMPA-IM users……53

Table 6: Multinomial logistic regression analysis of changes in immune mediator concentrations in LNG implant compared to DMPA-IM users……54

Summary

The Evidence for Contraceptive Options and HIV Outcomes (ECHO) trial found no substantial difference in HIV acquisition risk between women randomised to injectable depot medroxyprogesterone acetate (DMPA-IM), copper intrauterine device (IUD) or the levonorgestrel (LNG) implant. However, it remains unknown whether these contraceptives increase HIV risk relative to other forms of contraception or no contraception. This study investigated the impact of DMPA-IM, copper IUD and LNG implant on cervicovaginal inflammatory profiles previously associated with HIV acquisition, among a sub-cohort of ECHO participants.

This study included 167 ECHO participants at the Setshaba Research Centre in Pretoria and MatCH Research Unit in Durban, South Africa. Eleven cytokines and antimicrobial peptides were measured in lateral vaginal wall swabs in duplicate using Luminex. Differences in baseline cytokine profiles were assessed using demographic data, including site, age, body mass index (BMI) and sexually transmitted infection (STI) status. Changes in cytokine concentrations were assessed using Wilcoxon signed ank test. Fold changes in cytokine concentrations were compared between arms using Mann-Whitney U test. P-values were adjusted for multiple comparisons using a false discovery rate procedure. Overall cytokine profiles were compared using principal components analysis and unsupervised hierarchical clustering. Mixed effects linear regression was used for longitudinal analysis and multinomial logistic regression was used to adjust for potential confounders that were assessed at baseline.

Concentrations of IL-6 and MIP-3α were significantly higher in women enrolled at the Setshaba Research Centre compared to the MatCH Research Unit. Several immune mediators were elevated in younger women and this trend was significant for the pro-inflammatory IL-1β and the chemokines IL-8 and MIP-1α. Women that were seropositive for herpes simplex virus type 2 (HSV-2) had significantly lowerconcentrations of MIP-1α. The copper IUD and LNG implant were associated with rapid increases in inflammatory markers following contraceptive initiation. Pro-inflammatory IL-1β and IL-6 and chemotactic IL-8, IP-10, MIP-1α and MIP-1β were significantly elevated one month following copper IUD insertion. No changes were evident at one-month post LNG implant insertion, however at three months, TNF-α, IP-10, MIP-3α and SLPI were significantly raised relative to baseline. No significant changes in immune mediator concentrations were detected following DMPA-IM initiation but the trend was towards a decrease, particularly for SLPI. After adjusting for potential confounders, including site, age and infection status with chlamydia, gonorrhoea and HSV-2, IL-6 and IP-10 were significantly elevated in the copper IUD compared to the DMPA-IM arm at months 1 and 3, while IP-10 and SLPI were higher in the LNG implant arm at month 3 compared to the DMPA-IM arm.

The copper IUD and the LNG implant are associated with increased cervicovaginal inflammatory markers that have been linked to HIV infection risk and the chemokine IP-10 appears to play a central role. Recent studies have demonstrated the importance of the interplay between inflammation, the microbiome, contraception and HIV risk. Continued research to understand these effects are critical for safe contraceptive use and to inform novel contraceptive development.

Background

Introduction

With a total of 270 000 new HIV infections reported in South Africa in 2017, the country remains the most affected by the virus to this day[1]. Young women and adolescent girls, in particular, account for the large majority of incident infections in this region every year and it is estimated that they are twice as likely to acquire the virus as their male peers[1]. Although a portion of this high vulnerability among women in South Africa can be explained by certain socio-cultural behavioural patterns[2,3], it is now recognized that biological factors also play an important role[4–6].

Immunity in the Female Genital Tract

Transmission rates of HIV, in the case of unprotected receptive vaginal intercourse, are relatively low at an estimated 0.1% per coital act in healthy individuals[7]. This low rate of transmission is largely due to the cervicovaginal epithelium, a transition barrier that separates the external environment from the internal body, offering protection through both anatomical and biological components[8]. While the upper part of the genital tract, the endocervix, is covered by a single layer of columnar epithelium, the vagina and the ectocervix, which make up the lower genital tract, are characterized by the presence of stratified squamous epithelium[9]. Covering these surfaces is an additional layer of differentiated epithelial cells known as the stratum corneum[10]. The integrity of the epithelium is promoted by the presence of proteins known as tight and adherens junctions, which decrease the permeability of the barrier[11]. Interestingly, tight junctions in the lower reproductive tract are mostly found between basal epithelial cells and are absent from superficial layers, contributing to an environment which is more prone to penetration by pathogens, including HIV[12]. Covering mucosal surfaces is a layer of mucus, a major component that affects pathogen transmission. Mucus acts as a physical barrier[13] and contains a high concentration of soluble proteins, such as antimicrobial agents and antiproteases, which aid in the protection against pathogens[14,15]. Protection in the lower genital tract is further enhanced by the presence of the commensal microbiota which is dominated by *Lactobacillus* species in women with optimal microbiota[16]. Through the production of lactic acid, the commensal bacteria maintain the acidic pH of the mucus. The production of hydrogen peroxide as well as bacteriocins further contributes to the antagonistic interaction of *Lactobacillus* species with pathogenic bacteria and viruses by directly inhibiting their growth[17]. While the above-mentioned components are an effective first-line defence mechanism against invading organisms, genital inflammation occurs following infection or

outgrowth of pathogenic/non-optimal microbes. Epithelial cells are largely involved in the initiation of inflammatory responses that form part of the biological defence mechanisms of the female genital tract (FGT)[11]. The release of cytokines through activation of pattern recognition receptors forms a crucial part of this response as these soluble factors are involved in stimulating a response from a variety of immune cells[11].

Inflammation and HIV Risk

Although inflammation is an indispensable part of the immune response against pathogens and thus plays an important role in maintaining health, it has previously been shown that elevated levels of inflammatory cytokines in the FGT are linked to an increased risk of HIV acquisition[4,18,19]. Strikingly, the risk of HIV infection was three times higher in women with elevated levels of at least five of nine mucosal pro-inflammatory cytokines, including the macrophage inflammatory protein (MIP)-1α, MIP-1β, interferon gamma-induced protein (IP)-10, interleukin (IL)-8, monocyte chemoattractant protein (MCP)-1, IL-1α, IL-1β, IL-6 as well as tumor necrosis factor (TNF)-α[4]. In line with this, Mlisana, *et al*. also reported an association between higher concentrations of IL-1β, IL-6 and IL-8 in cervicovaginal lavage samples and an increased risk of HIV infection[20]. A recent study by Morrison, *et al*. showed that elevated levels of RANTES (regulated on activation, normal T cell expressed and secreted) as well as decreased levels of secretory leukocyte protease inhibitor (SLPI) were associated with HIV seroconversion[18]. A recent study further found that inflammation in the FGT significantly reduced the effectiveness of tenofovir gel in preventing HIV acquisition[19]. These findings suggest that some biomedical HIV prevention methods may be ineffective in the presence of genital inflammation and a deeper understanding of genital inflammation in the context of HIV risk is crucial.

HIV relies on the presence of immune target cells, specifically $CD4^+$ cells, to replicate as this is the entry receptor. In general, pro-inflammatory cytokines, as well as chemokines, facilitate the activation, differentiation and recruitment of these target cells, thereby creating favourable conditions for the virus[9]. More specifically, IP-10, MIP-1α, MIP-1β as well as RANTES are chemokines that have been found to increase HIV risk by recruiting HIV target cells[9,18,21]. MIP-1α, MIP-1β and RANTES bind directly to the C-C chemokine receptor type 5 (CCR5), one of the HIV coreceptors, and therefore specifically target $CCR5^+$ cells for recruitment to the genital mucosa[22]. In the context of sexual transmission, HIV infection is preferentially established by R5-tropic viruses, highlighting the critical role of this coreceptor[23]. It has thus been proposed that high concentrations of these chemokines in the FGT lead to the increased recruitment of

HIV target cells, in turn increasing the probability of infection. In addition, pro-inflammatory cytokines promote the expression of nuclear factor (NF)-κB. This influential transcription factor, in turn, directly binds to the HIV promoter and enhances its transcriptional capacity, thereby promoting productive infection further[24].

Much research has linked increased inflammation in the FGT to disturbances in epithelial barrier functions. Arnold, *et al.* showed that proteins associated with tissue remodelling processes, such as those involved in regulating the organization of the actin cytoskeleton, were up-regulated in individuals with an overall inflammatory profile[25]. Simultaneously, proteins that indicate mucosal barrier integrity, including a key marker of late epidermal differentiation known as cornulin[26], were found to be down-regulated. According to these results, a model was suggested in which inflammation leads to increased tissue remodelling at the expense of mucosal barrier integrity, suggesting another possible mechanism underlying the relationship between inflammation and heightened HIV risk[25]. Furthermore, neutrophil-associated proteins, especially certain proteases, were positively associated with inflammation. Linked to their role in endometrial degradation during menstruation[27], these proteases may be involved in disrupting the mucosal barrier and thus enhancing immune cell migration and increasing the access of HIV to target cells[25]. Further validating this theory is the result of a previous study which discovered an increase in antiproteases, belonging to the family of serpins and elafins, to be associated with HIV resistance in a cohort of sex workers from Kenya[28]. Additionally, a direct relationship between pro-inflammatory cytokines and disruption of epithelial barrier function has been demonstrated using *in vitro* models[29]. Sexual intercourse itself can result in micro-abrasions in the FGT mucosa, causing increased inflammation accompanied by increased wound healing processes and the recruitment of immune cells[30]. Exposure to semen further contributes to impaired immune function in the FGT. The pH of normal semen lies between 7.2 and 7.8, making it more alkaline than the acidic environment of the vagina in women with optimal microbiota[31]. Its presence therefore increases the vaginal pH. The acidic mucus of the vagina has been shown to slow the rate of HIV diffusion by interfering with the negatively charged surface of the virus. A shift to a more alkaline environment allows HIV to retain its negative charge, resulting in a higher rate of diffusion and likelihood of infection[32,33]. Additionally, semen exposure increases levels of pro-inflammatory cytokines, including IL-6, IL-8 and IL-1β, and has been associated with an increase in local HIV target cells[34,35].

The Role of Bacterial Vaginosis and Sexually Transmitted Infections in Genital Inflammation and HIV Risk

Sexually transmitted infections (STIs) have been identified as major causes of elevated cytokine concentrations in the genital tract[36]. Both viral infections, such as herpes simplex virus 2 (HSV-2), and bacterial infections, including *Chlamydia trachomatis and Neisseria gonorrhoeae*, are highly prevalent in sub-Saharan Africa, most likely contributing to the disproportionate vulnerability of this population to HIV[37]. Adolescent females in South Africa bear a particularly high burden of STIs, with studies reporting rates of *C. trachomatis* prevalence of up to 42%[38]. HSV-2 has been identified as the most influential infection with regards to HIV vulnerability. In a study conducted by Masese, *et al*., the overall risk of HIV infection in a cohort of African women was predominantly attributed to HSV-2 infection[5]. While ulcerative lesions directly disrupt the mucosal barrier, HIV target cell concentrations at the mucosa are increased during HSV-2 infection, regardless of the presence of lesions[39]. Additionally, subclinical levels of inflammation in the genital mucosa can persist for months after lesion resolution[40].

Although not considered to be an STI, bacterial vaginosis (BV) is a sexually-associated condition that causes high levels of inflammation in the FGT[41]. BV occurs when the genital microbiota shifts from predominantly lactobacilli to a more diverse, non-optimal population including anaerobic *Gardnerella, Prevotella* and *Atopobium* species[42]. Younger women generally have a greater prevalence of these anaerobic bacteria, while more favourable bacteria become more abundant with age[43]. Clinically, BV is defined using the Amsel criteria[44] or the Nugent score[45]. The Amsel criteria is based on the presence of several clinical signs, including a vaginal pH higher than 4.5, detection of fishy odour and the presence of clue cells. Nugent scoring involves Gram staining of a vaginal smear to quantify *Lactobacillus, Mobiluncus* and coccobacilli. Scores range from optimal (0-3), through intermediate (4-6), to BV positive (7-10). However, the development of high-throughput sequencing techniques, such as 16S rRNA sequencing, has enabled more detailed characterisation of bacterial populations in the FGT compared to these traditional methods[46]. Dysbiosis is often difficult to define and some BV-associated bacteria are detected in BV negative women. For example, *Gardnerella vaginalis* is a bacterium that can be present as a commensal and is only involved in disease when present in abnormally high abundance[47]. In addition, four different subtypes of the bacterium have recently been identified and their prevalence among BV positive women varies. While clade one, two and three are prevalent in women with BV, clade four *G. vaginalis* is commonly seen in women without BV[48].

The vaginal microbiota differs greatly across individuals and may be influenced by many factors, including ethnicity[49]. A recent study showed that the HIV acquisition rates of young South African women with highly diverse genital microbiota and a low abundance of *Lactobacillus* species were over four-fold higher than observed in women with *L. crispatus* dominance[50]. Furthermore, the above described microbial community type, including diverse genital microbiota and a low abundance of *Lactobacillus* species, was associated with elevated cytokine levels as well as an increased proportion of HIV target cells in the FGT. Due to the frequently asymptomatic nature of the condition, BV oftentimes remains undiagnosed and even if treatment occurs, recurrent episodes are common[51]. Compared to the 90% of women in developed countries who have *Lactobacillus*-dominant vaginal communities, South African women frequently have low *Lactobacillus* abundance coupled with a lack of clinical BV symptoms[38]. Furthermore, if the vaginal microbiome of South African women is dominated by *Lactobacillus* spp., it commonly tends to be *L. iners*[52]. *L. iners* is frequently present in women with non-optimal vaginal microbiota and whether it plays a protective role in the FGT remains uncertain[53]. Compared to other *Lactobacillus* species, *L. iners* has a smaller genome and it has been speculated that this could be indicative of a more symbiotic or parasitic relationship with its host[53]. Additionally, the genome of *L. iners* encodes inerolysin, a pore-forming toxin that is related to vaginolysin, produced by *G. vaginalis* [53]. This has led to the belief that beyond a lack of protection, this specific species of *Lactobacillus* may even be involved in the onset and persistence of BV[54]. Importantly, even when BV is asymptomatic, it causes dramatic increases in pro-inflammatory cytokines, although down-regulations of some mediators, including chemokines, may occur[55].

The Role of the Hormonal Cycle in Inflammation and HIV Risk

While there is substantial evidence supporting the concept that STIs and BV shift the vaginal environment to a more pro-inflammatory milieu, the role of sex hormones, whether endogenous or synthetic, remains controversial. Sex hormones are heavily involved in the regulation of immunity in the FGT by maintaining the balance between protection from pathogens and at the same time allowing for the reproductive functions of the FGT. This delicate balance is largely facilitated by oestradiol and progesterone, two hormones produced periodically by the ovaries[56]. The menstrual cycle itself can be divided into four stages: the menstrual phase, the proliferative (or follicular) phase, the mid-cycle and lastly, the secretory (or luteal) phase. Oestradiol levels rise throughout the proliferative phase and peak before mid-cycle. This is followed by a rapid increase in luteinizing hormone, which is needed for

ovulation to occur. Peak concentrations of progesterone are observed at the mid-secretory phase. The fluctuation of hormone levels is thought to play a role in HIV acquisition risk. More specifically, the secretory phase of the menstrual cycle has previously been referred to as the "window of vulnerability", during which women are thought to be at a higher risk of contracting the virus[56]. This theory is based mostly on epidemiological observations and results differ greatly with some supporting[57] and others opposing the theory[58].

Interestingly, a recent study conducted by Villegas, *et al.* showed that ectocervical tissues obtained from women in the secretory phase of the menstrual cycle had an overall higher pro-inflammatory profile than those harvested during the proliferative phase, with several important inflammatory markers, including IL-6, IL-7 and IL-8, elevated[59]. These changes are potentially relevant to HIV transmission as it has been shown that IL-6 increases HIV expression *in vitro* [60], while high levels of IL-8 increased HIV infection up to 8-fold in cervical explant tissue[61]. Additionally, observational studies have linked high concentrations of genital IL-6 and IL-8 to an increased risk of HIV infection[4,20].

Furthermore, HIV shedding in the presence of elevated IL-6 levels was heightened in cervicovaginal lavage obtained from HIV-infected women[58]. High levels of IL-7, on the other hand, have been linked to increased CD4+ T cell proliferation as well as inhibited T cell apoptosis in ectocervical explants. This, in turn, increased the availability of HIV target cells, thus risk of infection when tissues were exposed to the virus[62]. Although the role of IL-8 in HIV risk is less well established, generally the cytokine has chemotactic effects on neutrophils and macrophages and thus increases inflammation and target cell availability[63,64]. Interestingly, IL-8 signalling was one of the most notable pathways associated with the secretory phase of the menstrual cycle[61,65]. This suggests that this cytokine, among others, may play a crucial role in the context of HIV acquisition. Additionally, progesterone is able to alter the humoral immunity of an individual, with reports demonstrating decreased IgG and IgA concentrations in vaginal secretions during the luteal phase[66]. The secretory phase of the menstrual cytokine has also been associated with reduced epithelial barrier function. Birse *et al.*, recently demonstrated an association between proteins involved in tissue remodelling and the luteal phase of the menstrual cycle. Cell-to-cell adhesion proteins as well as certain anti-proteases were reduced during the secretory phase, indicating that epithelial barrier function may play a role in hormone-related mechanisms of increased HIV susceptibility[65]. Furthermore, thinning of the vaginal epithelium has been observed in response to high doses of progesterone during

the luteal phase of the menstrual cycle in primates[67]. These changes may alter susceptibility to HIV acquisition.

Contraceptives and HIV risk

Contraception has many benefits, including reduced maternal and infant mortality and morbidity, the empowerment of women as well as a reduction in the number of HIV-infected children[68]. A wide variety of Long-acting hormonal and non-hormonal contraceptive methods are currently available and represent a popular choice among women due to their longevity and low failure rate[69]. These include: 1) rings, 2) implants, 3) IUDs (intrauterine devices) and 4) injectables. Combined oral contraceptive (COCs) pills and IUDs are two of the most prevalent HCs used by women worldwide[70]. In 2015, approximately 14% of married or in-union women worldwide relied on an intrauterine device for protection, while 9% used COCs[70]. Studies have shown that COCs may have beneficial effects on the vaginal microbiome by increasing *L. crispatus* abundance and thus decreasing rates of BV[71]. While some women in low- and middle-income countries use COCs, the majority rely on long-acting injectables for contraception[70].

Injectables

Injectables are a popular choice among hormonal contraceptives (HCs). In South Africa, approximately 24% of women use injectables with the three-monthly intramuscular injection of 150 mg depomedroxyprogesterone acetate (DMPA-IM) being the most popular choice[70]. To this day, high rates of intimate partner violence, especially in the form of female oppression, drive the high use of this injectable due to its relative longevity and discrete administration[72]. While one of the most popular choices, DMPA-IM is not the only method of progestin-based long-acting HC, with other types varying in the dose and type of progesterone-like compounds, synthetic analogues of the endogenous hormone progesterone. As increases in progesterone levels after ovulation cause changes in the endometrium that facilitate egg implantation, the use of synthetic analogues transforms the endometrium into secretory phase tissue and ovulation is prevented. Additionally, thinning of the endometrium is initiated and glycogen secretion is reduced, causing the cervical mucus to become thicker and more viscous and thus preventing sperm penetration and the implantation of embryos[73].

The frequent usage of DMPA-IM in South Africa overlaps with the disproportionally high burden of HIV observed in these women and epidemiological data suggests that use of the injectable may increase HIV risk by 40-50%[74,75]. However, the contribution of HCs, in particular DMPA-IM, to HIV risk is controversial among scientists[76]. Recently a large

randomized control trial, the Evidence for Contraceptive Options and HIV Outcomes (ECHO), was conducted to further evaluate the risk of HIV infection associated with the use of DMPA-IM. The results of the trial did not demonstrate statistically significant differences in HIV risk between women using 150-IM compared to those using the copper intrauterine device (Cu-IUD) or the levonorgestrel (LNG) implant. However, the study was only powered to detect an increased risk above 50%. An analysis adjusted for covariates suggested that the use of DMPA-IM may increase HIV risk by 29% compared to the LNG implant [hazard ratio: 1.29 (0.98 – 1.71, p=0.060)], which would contribute substantially to HIV incidence[77]. It therefore remains unknown whether these contraceptives increase HIV risk relative to other forms of contraception or no contraception[78].

Several studies have suggested the possible effect of DMPA on HIV risk may be dependent on the dose administered[73]. Interestingly, different contraceptives differ greatly with regards to serum concentration, both at peak and trough levels. The dosage of progestins, route of administration, as well as the metabolism of the patient are only three of many factors that influence serum levels at any given time. In the context of increased HIV risk, it is highly important to note that no information is available on tissue concentrations of progestins. Sayana Press, also known as DMPA-SQ or DMPA-SC, is a lower dose formulation (104 mg) which is administered in a subcutaneous manner. It has become apparent that the 150 mg dose of DMPA usually administered is extremely high and the 31% lower dose delivered in DMPA-SQ still inhibits ovulation[79]. As this lower dose formulation has potential benefits compared to DMPA-IM, its introduction is predicted to increase DMPA usage worldwide[73]. Norethisterone enanthate, usually referred to as NET-EN or Nur-Isterate, is another popular injectable contraceptive. Similar to DMPA, it is injected intramuscularly every two months and is primarily used in South Africa. An intramuscular dose of 200mg NET has similar contraceptive efficacy to DMPA-IM[80].

In response to increasing epidemiological evidence and rising concerns, the World Health Organization changed the criteria for the use of DMPA-IM, DMPA-SC and NET-EN from no restriction (medical eligibility criteria for contraceptive use category 1) to category 2, indicating that the benefits generally outweigh the risks. However, based on the results of the ECHO trial, which did not report on an increased risk of HIV associated with DMPA-IM use when compared to the LNG implant and non-hormonal copper IUD, these contraceptives have now been moved back to category 1. Despite this, the possible contribution of these contraceptives to HIV risk remains controversial among researchers in the field[81]. Additionally,

aside from the question of altered HIV risk, it remains unknown whether these contraceptives cause vaginal microbiome and immune changes that may influence reproductive health. Associations between HC use and immunological factors, the microbiome and HIV risk thus warrant further investigation.

Intrauterine devices

IUDs are amongst the most popular contraceptive methods, with the copper IUD having an efficacy of > 99%[82]. Data evaluating associations between copper IUD use and HIV risk are extremely limited and weak[83]. Although two older studies have suggested a possible link[84,85], the majority of existing reports indicate that the copper IUD does not increase a woman's risk of acquiring the virus[86–88]. It is important to note that there are no recent studies that have investigated further.

Implants

Levonorgestrel (LNG) implants represent an additional form of HC. Data for these types of contraceptives suggests their usage does not significantly increase the risk of HIV acquisition, however there is currently minimal data [74]. Similar to the copper IUD, data on HIV risk in implant users are sparse. Early studies in rhesus macaques suggested that progestin-only implants enhance transmission of Simian Immunodeficiency Virus (SIV) and early viral load[89]. More recent studies, however, have not identified a link between an increased HIV transmission risk and the use of implant contraceptives, specifically the LNG implant that is typically sold under the name Jadelle[90].

Mechanisms underlying the possible relationship between contraceptives and HIV risk

Impact of long-acting contraceptives on FGT immunity

Progestins exert their function by interacting with a variety of steroid receptors and thereby altering downstream gene expression. The most important receptor in this context is the progesterone receptor (PR), however, progestins also interact with the glucocorticoid receptor (GR), as well as the mineralocorticoid receptor (MR), among others[91]. Although progestins are generally designed to act like progesterone, different progestins interact with members of the steroid receptor family to varying degrees and thus cause differential effects. Overall it is important to note that all progestins have a higher affinity for the PR in comparison to endogenous progesterone[73,92]. Interestingly, the progestin MPA seems to be an outlier with regards to steroid receptor binding. Unlike NET-EN and its endogenous counterpart progesterone, it has a high affinity for the GR. On the contrary, while progesterone has a high affinity for the MR, MPA has a low affinity[73,93]. Although these relationships have been

established, the downstream effects of this differential binding are largely unknown. It is also important to note that the affinity of an analyte is not necessarily directly proportional to biological activity, however, as MPA is a clear outlier in this regard, it is worth investigating further.

It is well documented that high doses of MPA (approx. 1000 mg/day) have immunosuppressive effects and are frequently used to treat cancer patients undergoing therapy[94]. Although these effects have been observed in previous studies, the cut-off concentration, below which immunosuppression is possibly no longer observed, as well as its application to immunity in the FGT, remains unknown. In line with the possibility of immunosuppressive properties, Ngcapu, *et al*. reported that the FGTs of women using injectables had an overall less inflammatory profile than women not using injectables[95].

Contrary to these findings, numerous studies describing the inflammatory potential of the hormone progestin and its synthetic analogues have emerged, specifically with regards to the FGT. Calla *et al*. recently demonstrated that DMPA, as well as LNG, usage was associated with tissue inflammation in the FGT. The authors established a model whereby decreased levels of the desmosomal cadherin desmoglein-1a lead to increased mucosal permeability and thus facilitated the invasion of endogenous vaginal bacteria, increasing genital inflammation overall[96]. Furthermore, it has been demonstrated that cervical cells obtained from women using DMPA contained higher numbers of activated HIV target cells. On the contrary, women using IUDs as a method of contraception had reduced expression of the CCR5 co-receptor on CD4+ T cells in cervical cells[97]. Morrison *et al*. showed that DMPA users had higher levels of RANTES in their genital tracts, which was also linked to higher risk of HIV acquisition in the same study[18].

The possible biological link between long-acting contraceptives and HIV risk is thus likely multifactorial, with increased or decreased genital inflammation playing a central role. Furthermore, if there are concentration ranges which determine whether the effects of DMPA are anti- or pro-inflammatory, these are unknown and should be investigated. The uncertainty around the effects of DMPA on FGT immunity is further highlighted in a recent study by Zalenskaya *et al*. in which the authors assessed the effects of DMPA on gene expression in ectocervical tissues. Pathway analyses demonstrated that predicted alterations of the transcriptome correlated with an immunosuppressive effect of DMPA, however, in some women they were more compatible with an inflammatory-like response[98].

A recent study reported that NET-EN use was not linked to an increase in FGT cytokine levels when compared to combined contraceptive vaginal rings[99]. Contrary to this, Dabee *et al.* detected an upregulation of 33/44 genital cytokines in women using DMPA-IM, as well as NET-EN[100]. Another study has linked NET-EN use to increased levels of IL-6, IL-8 and RANTES concentrations in the FGT[101]. It becomes clear that studies assessing genital inflammation following the use of NET-EN and DMPA-IM have produced diverse results and more research is needed to determine how injectables may influence HIV acquisition risk.

Impact of contraceptives on cervicovaginal structure

Early evidence for the possible association between progesterone and HIV emerged from studies in non-human primates that showed that implants containing 200 mg of progesterone increased the susceptibility to SIV infection following vaginal inoculation[102]. Furthermore, MPA has been administered intramuscularly in doses comparable to those given to women for contraceptive purposes to facilitate infection with STIs in mouse and non-human primate models[103]. Thinning of the vaginal epithelium was proposed as the potential underlying mechanism[102]. In primate models, this is commonly observed in response to high doses of progesterone (i.e. during the luteal phase of the menstrual cycle as discussed earlier[67]) or its synthetic analogue progestin, as found in the contraceptive DMPA[102]. Whether these changes can be directly translated to humans is currently less clear and debated among scientists[104,105]. Zalenskaya *et al*., supported this theory in a study that assessed differences in gene expression in ectocervical tissues between women using DMPA and COCs. The expression of 235 genes was significantly altered and mostly downregulated in response to DMPA usage, with the majority involved in maintenance of mucosal barrier integrity[98].

Impact of synthetic hormones on endogenous hormones

While most reports focus exclusively on the effects of progesterone and its synthetic analogue on HIV risk, Villegas, *et al*., recently investigated the importance of oestradiol in the protection against HIV infection[59]. Most importantly, an inverse relationship between serum concentrations of oestradiol and efficiency of HIV infection of endo- and ectocervical tissue *ex vivo* was established. This suggests that, contrary to progesterone and its analogues, oestradiol plays a protective role in the context of HIV infection. Different forms of HC may have different effects on endogenous levels of oestradiol. A comparison of serum oestradiol levels for different forms of HCs has revealed that DMPA-IM users are typically in a hypoestrogenic state[105]. Hypoestrogenism may reportedly have effects on epithelial integrity, the FGT microbiota and immune function in the FGT as a whole and may thus be crucial in the context of HIV risk[106]. It is important to note that DMPA-IM is a progestin-only contraceptive and, unlike some other HCs, such as most COCs, contains no synthetic analogue of oestrogen.

HCs and the acquisition of STIs and BV

The immunosuppressive properties of high DMPA doses are thought to leave the FGT vulnerable to infection with genital pathogens, including BV and STIs other than HIV. However, the evidence for potential associations between the use of HCs and risk of STIs aside from HIV is sparse, with few studies investigating further. STIs have been identified as a major cause of elevated cytokine concentrations in the FGT[36], increased HIV risk and increased risk of reproductive complications. Therefore, the impact of long-acting HCs on the risk of STI acquisition has important public health implications.

Two studies have recently suggested a link between oral contraceptive use and chlamydia infections but not other STIs, including gonorrhoea, HSV-2, trichomoniasis, syphilis, and human papillomavirus (HPV)[83,107]. Interestingly, COC use was also associated with the development of cervical ectopy, which in turn increases the risk of chlamydia, HPV, as well as HIV infection[73]. Additionally, studies in mice have shown a 2-fold increase in HSV-2 infection when treated with DMPA[108].

Information on the effects of DMPA-IM on the vaginal microbiome is limited and studies have reported highly inconsistent results. Cultivation-based longitudinal studies have shown a decrease in H_2O_2-producing *Lactobacillus* spp., both 6 and 12 months after initiation of DMPA use. In line with this, low oestrogen levels that are associated with the use of progestin-only contraceptives correlated with decreased growth of *Lactobacillus* spp. and increased growth of BV-associated bacteria in the FGT[109]. Meanwhile, the use of HCs that are formulated with

oestrogen has been linked to a decrease in BV-associated bacteria in the FGT[109]. Oestrogen induces the accumulation of glycogen in the vaginal epithelium and it is thus thought that synthetic oestrogen-containing contraceptives positively influence colonization by *Lactobacillus* spp. (that utilise glycogen as a food source) and therefore confer protection from BV[110].

Contrary to the results described above, Roxby *et al.* observed a decrease in *G. vaginalis* growth alongside an increase in *L. crispatus* numbers following DMPA-IM initiation [111]. However, a study with a similar design observed a decrease in *L. iners* growth but no effect on more beneficial *Lactobacillus* strains[110]. Additionally, it has been suggested that the effects observed in response to DMPA are dependent on the ethnicity of the user. Yang *et al*. demonstrated that while black women showed an increased colonization of BV-associated bacteria in their FGTs, the same was not observed in Hispanic and white women following the use of the HC[112].

Additionally, there are conflicting results regarding current data evaluating the risk of acquisition of STIs other than HIV in IUD users. For example, while some studies reported an increase in risk of *C. trachomatis* infection in current IUD users[113], others failed to establish this link[114]. Interestingly, it has been suggested that the copper IUD can affect the vaginal microbiome. A recent study demonstrated that the prevalence of BV in a cohort of copper IUD users increased from 27% at baseline to 35% at 30 days and reached 49% at 180 days following initiation[110]. Specifically, two key BV-associated bacterial spp., *G. vaginalis* and *A. vaginae,* showed an increase in growth. Similarly, Brooks *et al*. found a number of taxa typically associated with a dysbiotic vaginal microbiome, including *Prevotella* spp., *Sneathia amnii* and *Megasphaera* spp., to be significantly more abundant in women using the LNG-intrauterine system (IUS), more commonly known as the Mirena[109]. The results linking IUDs/IUSs to a higher risk of developing vaginal dysbiosis could have important public health implications with regards to HIV risk in IUD users and further investigations are crucial.

Studies assessing the risk of the acquisition of other STIs in implant users are somewhat more available but also limited. Studies by Maucort-Boulch *et al*. and McClelland *et al.* reported a lack of association between use of the LNG implant and HPV persistence as well as BV, respectively[115,116]. On the contrary, a study of a Ugandan cohort found that LNG implant use was associated with higher rates of trichomonas infection when compared to women not using any form of HC[117]. Furthermore, a study conducted in sub-Saharan Africa linked the use of

LNG implants to a higher risk of gonorrhoea, but not syphilis[85]. Overall these results are inconclusive and largely based on observational studies.

Rationale

Cytokine profiles in the FGT are thought to play an important role in the HIV risk of individuals. The effects of HCs on these cytokine profiles (and by extension HIV risk) requires further attention as part of the investigation into biological mechanisms that facilitate the disproportionally high burden of HIV infections in women. Additionally, aside from the question of altered HIV risk, it remains unknown whether certain contraceptives cause vaginal microbiome and immune changes that may influence reproductive health.

Aims

1. To investigate changes in cytokine signatures associated with the initiation of DMPA-IM and compare these to LNG implant and copper IUD insertion.
2. To evaluate baseline demographic and biological factors that may alter cytokine responses to DMPA-IM, LNG implant and copper IUD.

Methods and Materials

Study participants

The impact of two progestins (MPA and LNG) and the copper IUD on the cytokine signatures in the FGT tract were evaluated in this study. FHI 360 collected specimens as part of the ECHO (Evidence for Contraceptive Options and HIV Outcome) study[78]. ECHO was a randomized controlled trial to evaluate the HIV incidence among 7800 women randomized to 150 mg intramuscular DMPA, the LNG implant or the copper IUD in South Africa, Kenya, Zambia and Swaziland. The samples included in the present study were collected at two sites in South Africa, Setshaba Research Centre (SRC) in Pretoria (n=54) and MatCH Research Unit (MRU) in Durban (n=113). Specimens were collected at baseline, i.e. immediately prior to method initiation, month 1 (M1) and month 3 (M3) in order to facilitate immune assessment at near peak and near trough MPA concentrations. Demographic and behavioural data were collected by questionnaire.

Specimen collection

Lateral vaginal wall swabs were collected at each visit for measurement of cytokines and secretory leukocyte protease inhibitor (SLPI) concentrations. Dacron swab samples were collected by placing the swabs on the lateral vaginal wall and rotating 360 degrees. Swabs were placed in cryovials and stored at -80°C. All collected samples were kept at 4ºC immediately after collection and transported to the clinic laboratory for storage within 4 hours of collection.

STI diagnosis

STI diagnosis was conducted at Bio Analytical Research Corporation South Africa (BARC SA) and included detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* using GeneXpert and herpes simplex virus type 2 serology[78].

Lateral vaginal wall swab processing and aliquoting

The frozen lateral vaginal wall swabs were thawed on ice overnight at 4ºC. The following day, 1 mL of phosphate buffered saline (PBS; Sigma-Aldrich, P5493) was added to each tube containing a swab. The tubes were vortexed for 60 sec at a low speed and subsequently incubated at 4ºC for 1 hour. Holding the swab, excess mucus was scraped off on the wall of the tubes and each tube was vortexed for 30 sec at a low speed. The supernatants were transferred into filter centrifuge tubes (Corning® Costar® Spin-X® tubes Sigma-Aldrich, CLS8160) and centrifuged for 10 min at top speed (4000 rpm). Filters were removed and the supernatants vortexed for 10 sec at a low speed prior to cytokine/SLPI measurement. The tubes were kept on dry ice throughout this protocol.

Cytokine, antimicrobial peptide and prostate specific antigen measurement

Using lateral vaginal wall swabs collected as part of the ECHO trial, pro-inflammatory cytokines and chemokines including MIP-1α, MIP-1β, MIP-3α, IP-10, RANTES, IL-6, IL-8, IL-1β and TNF-α, innate immune mediator IFN-α and antimicrobial peptide SLPI were measured using Human Magnetic Luminex Screening Assays (R&D Systems, Minneapolis, USA. Lot L128368; supplementary figure S1). The assay principle is explained in detail in the appendix (figure S1). Samples were analysed on a Bio-Rad Bio-Plex® 200 system with Bio-Plex Manager Software 6.1 (Bio-Rad, Hercules, CA) as described previously[4]. Cytokines were measured in duplicate and the averages of the duplicates were used for downstream analysis. Samples were run over 13 plates for each, the larger panel (MIP-1α, MIP-3α, IP-10, IL-6, IL-8, IL-1β, TNF-α, IFN-α and SLPI) and the smaller panel of cytokines (MIP-1β and RANTES). Examples of standard curves for each immune mediator are shown in the Appendix (figure S2). Prostate specific antigen (PSA) was measured by Mr Rushil Harryparsad using Human Kallikrein 3/PSA Quantikine ELISA (R&D Systems, US) to determine the presence of semen contamination.

Data analysis

Differences in median cytokine concentrations were assessed based on baseline demographic data, namely site, age group, body mass index (BMI), *Chlamydia trachomatis* status, *Neisseria gonorrhoeae* status, HSV-2 status as well as PSA presence/absence (using Mann-Whitney U test, Kruskal-Wallis test and Fisher's Exact test; GraphPad Prism). Changes in cytokine concentrations among the different contraceptive methods were assessed using Wilcoxon signed rank test at one- and three-months post contraceptive initiation. Differences in fold changes in cytokine concentrations between the contraceptive methods at one- and three-months post contraceptive initiation were assessed using Mann-Whitney U test. P-values were adjusted for multiple comparisons using a false discovery step down procedure. Principal component analysis (PCA) was used to group pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) and chemokines (IL-8, MIP-1α, IP-10, MIP-3α, MIP-1β and RANTES) and to generate estimates for each group. Differences in the magnitude of change of these PCA estimates between the contraceptive methods were assessed using Mann-Whitney U test. Additionally, PCA was performed on $\log_{10}$-transformed fold change values of immune mediator concentrations at one- and three-months following initiation of contraceptive method using the mixOmics package in R (R Foundation for Statistical Computing, Vienna, Austria). Heat maps of the $\log_{10}$-transformed fold changes in immune mediator concentrations were generated using

R. Mixed effects linear regression analysis using the log_{10}-transformed immune mediator concentrations was used to assess changes over time (STATA™, StataCorp). Multinomial logistic regression analysis using the log_{10}-transformed fold change data was used to assess the effects of possible confounders, namely site and age as well as gonorrhoea, chlamydia and HSV-2 status on immune mediator concentrations (STATA™).

Results

Baseline Demographics

The concentrations of eleven immune mediators were measured in lateral vaginal wall swabs from 167 women randomised to the injectable DMPA-IM (n=53), the copper IUD (n=55) or the LNG Implant (n=59). The relationships between baseline demographic data and immune mediator concentrations were evaluated to identify potential confounders (table 1). Concentrations of the pro-inflammatory cytokine IL-6 and the chemokine MIP-3α were significantly higher in women from SRC when compared to those from MRU. No significant differences in STI prevalence across the two sites was detected (figure 1). While PSA levels, as a biomarker of recent unprotected sexual intercourse, were significantly higher in women from MRU at baseline visit, women from SRC showed higher concentrations of the protein at both follow-up visits (figure 2). Women in the age groups of 18-20 and 21-24 years tended to have elevated concentrations of TNF-α, IL-1β, IL-8, MIP-1α, IP-10, SLPI and MIP-1β when compared to the ages 25-30 and 31-35 years. This trend was statistically significant for the pro-inflammatory IL-1β and the chemokines IL-8 and MIP-1α after adjusting for multiple comparisons. No significant differences in age were found between the two sites (figure S9). Immune mediator concentrations were generally higher in women with a BMI ≤ 30 compared to those with a BMI > 30, however statistical significance was not observed. All immune mediators, with the exception of MIP-1β, were present at lower concentrations in HSV-2 seropositive women but this trend was only statistically significant for the chemokine MIP-1α after adjusting for multiple comparisons. Infection with *C. trachomatis* had a diverse effect on immune mediator concentrations with some, including IL-1β, IL-8, MIP-1α, IP-10 and MIP-1β slightly raised in women who tested positive for the pathogen. Others, namely TNF-α, IL-6, MIP-3α, IFN-α and SLPI, were detected at lower levels in infected women, however, none of these associations remained significant after adjusting for multiple comparisons. Levels of immune mediators were generally higher in women infected with *N. gonorrhoeae*, particularly, IL-6 and IL-8 but this trend was not statistically significant after adjusting for multiple comparisons. The presence of PSA was not associated with any significant trends in immune

mediator concentrations, but levels of IL-6 and MIP-3α were slightly raised in the presence of this protein.

Table 1. Baseline demographic and clinical characteristics by immune mediator

	TNF-α (n=167)		IL-6 (n=167)		IL-1β (n=167)	
	median (range)	p value	median (range)	p value	median (range)	p value
Site: Setshaba Commercial City (n=54)	16.66 (86.29)	0.0352	14.19 (336.7)	0.0028*	30.49 (4027)	0.3725
Site: MatCH Research Centre (n=113)	12.65 (70.89)		10.44 (137.2)		32.27 (2386)	
	median (range)	p value	median (range)	p value	median (range)	p value
18-20 (n=33)	16.06 (70.89)	0.2175	10.48 (69.85)	0.1204	50.13 (4024)	0.0032*
21-24 (n=72)	16.91 (86.29)		12.94 (337.6)		39.61 (2386)	
25-30 (n=43)	11.56 (37.58)		9.62 (155)		16.83 (696.4)	
31-35 (n=19)	13.07 (30.39)		11.17 (52.82)		24.52 (195.3)	
BMI	median (range)	p value	median (range)	p value	median (range)	p value
≤ 30 (n=30)	15.95 (86.29)	0.0632	11.41 (340.3)	0.1898	31.85 (2386)	0.2035
> 30 (n=54)	11.18 (34.7)		10.45 (167.9)		24.20 (4027)	
HSV-2	median (range)	p value	median (range)	p value	median (range)	p value
Indeterminate (n=18)	11.19 (37.17)	0.0557	10.06 (67.24)	0.0822	26.63 (4026)	0.1153
Negative (n=91)	16.87 (86.29)		12.58 (337.6)		35.86 (2386)	
Positive (n=56)	11.11 (81.94)		10.11 (173.3)		19.47 (915.5)	
Chlamydia trachomatis	median (range)	p value	median (range)	p value	median (range)	p value
Negative (n=125)	15.95 (86.29)	0.0943	11.28 (173.3)	0.8752	29.21 (4027)	0.1780
Positive (n=42)	11.52 (70.14)		10.53 (338.1)		53.63 (1557)	
Neisseria gonorrhoeae	median (range)	p value	median (range)	p value	median (range)	p value
Negative (n=160)	14.44 (86.29)	0.7267	10.76 (430.7)	0.0234	30.51 (4027)	0.1166
Positive (n=7)	15.95 (30.2)		32.19 (158.6)		257 (1551)	
PSA	median (range)	p value	median (range)	p value	median (range)	p value
Negative (n=148)	15.62 (86.29)	0.3203	10.58 (340.3)	0.5473	31.6 (2386)	0.7141
Positive (n=19)	11.77 (23.99)		12.03 (33.19)		31.24 (4026)	

	IL-8 (n=167)		MIP-1α (n=167)		IP-10 (n=167)	
	median (range)	p value	median (range)	p value	median (range)	p value
Site: Setshaba Commercial City (n=54)	397.8 (29305)	0.9993	184.8 (854.1)	0.4986	15.08 (3924)	0.5120
Site: MatCH Research Centre (n=113)	469.8 (29310)		180.1 (718)		12.14 (3924)	
	median (range)	p value	median (range)	p value	median (range)	p value
18-20 (n=33)	867.2 (29305)	0.0075*	229.9 (569.9)	0.0008*	25.74 (78.39)	0.0515
21-24 (n=72)	522.7 (29310)		206.6 (854.1)		15.15 (3924)	
25-30 (n=43)	235.2 (29296)		110.8 (460.6)		8.91 (3924)	
31-35 (n=19)	329.4 (3048)		150.2 (590.5)		4.74 (197)	
BMI	median (range)	p value	median (range)	p value	median (range)	p value
≤ 30 (n=30)	536.7 (29309)	0.2034	206.3 (854.1)	0.0072	16.09 (317)	0.1258
> 30 (n=54)	244.0 (29310)		153.6 (576.9)		10.58 (3924)	
HSV-2	median (range)	p value	median (range)	p value	median (range)	p value
Indeterminate (n=18)	954.5 (29310)	0.0185	202.8 (359.6)	0.0016*	14.55 (94.8)	0.1748
Negative (n=91)	535.4 (29305)		206.3 (759.7)		16.02 (3924)	
Positive (n=56)	208 (29298)		139.1 (854.1)		8.565 (3924)	
Chlamydia Trachomatis	median (range)	p value	median (range)	p value	median (range)	p value
Negative (n=125)	407.6 (29310)	0.4372	181.9 (854.1)	0.405	13.94 (3924)	0.3264
Positive (n=42)	615.9 (29309)		184.5 (718)		15.02 (120.9)	
Neisseria gonorrhoeae	median (range)	p value	median (range)	p value	median (range)	p value
Negative (n=160)	422.8 (29310)	0.0523	182.3 (854.1)	0.1998	13.91 (3924)	0.4529
Positive (n=7)	1120 (29219)		193.2 (205.8)		16.84 (93.29)	
PSA	median (range)	p value	median (range)	p value	median (range)	p value
Negative (n=148)	451.7 (29310)	0.5423	184.1 (854.1)	0.7972	14.14 (3924)	0.6427
Positive (n=19)	451.1 (29287)		172.2 (334.4)		12.55 (118.2)	

	MIP-3α (n=167)		IFN-α (n=167)		SLPI (n=167)	
	median (range)	p value	median (range)	p value	median (range)	p value
Site: Setshaba Commercial City (n=54)	28.04 (255)	0.0026*	23.66 (91.54)	0.1426	70685 (1411682)	0.0375
Site: MatCH Research Centre (n=113)	14.31 (117.4)		20.11 (60.73)		34848 (1413049)	
	median (range)	p value	median (range)	p value	median (range)	p value
18-20 (n=33)	23.72 (167.5)	0.4585	22.85 (53.44)	0.2465	44315 (1411313)	0.4096
21-24 (n=72)	23.22 (255)		20.90 (93.88)		65913 (1413013)	
25-30 (n=43)	10.30 (124.1)		16.54 (39.12)		39695 (621985)	
31-35 (n=19)	11.90 (81.26)		23.37 (51.88)		32844 (714834)	
BMI	median (range)	p value	median (range)	p value	median (range)	p value
≤ 30 (n=30)	22.27 (255)	0.4214	21.59 (93.88)	0.1314	63757 (1413013)	0.1241
> 30 (n=54)	13.14 (90.11)		18.43 (55.22)		28388 (714834)	
HSV-2	median (range)	p value	median (range)	p value	median (range)	p value
Indeterminate (n=18)	29.18 (106.9)	0.1418	18.52 (73.81)	0.1	24445 (622125)	0.2637
Negative (n=91)	22.27 (255)		21.65 (62.98)		63663 (1413013)	
Positive (n=56)	11.17 (124.1)		18.03 (96.78)		29465 (1413049)	
Chlamydia Trachomatis	median (range)	p value	median (range)	p value	median (range)	p value
Negative (n=125)	19.21 (255)	0.8241	21.03 (96.78)	0.5338	55652 (1413049)	0.1296
Positive (n=42)	14.98 (106.9)		18.42 (45.95)		31322 (1411209)	
Neisseria gonorrhoeae	median (range)	p value	median (range)	p value	median (range)	p value
Negative (n=160)	17.55 (255)	0.2386	20.48 (96.78)	0.7206	45657 (1413049)	0.5329
Positive (n=7)	30.31 (106.9)		21.05 (43.77)		73094 (241809)	
PSA	median (range)	p value	median (range)	p value	median (range)	p value
Negative (n=148)	15.21 (255)	0.2658	20.9 (96.78)	0.2595	54996 (1413049)	0.4825
Positive (n=19)	30.31 (69.73)		18.75 (25.19)		26811 (1410232)	

	MIP-1β (n=167)		RANTES (n=167)	
	median (range)	p value	median (range)	
Site: Setshaba Commercial City (n=54)	226.5 (946.6)	0.7307	3.485 (419.5)	0.0141
Site: MatCH Research Centre (n=113)	122.6 (1114)		3.485 (609.4)	
	median (range)	p value	median (range)	
18-20 (n=33)	292.2 (946.6)	0.3056	3.485 (291)	0.3415
21-24 (n=72)	177.7 (1114)		3.485 (609.4)	
25-30 (n=43)	39.66 (886.5)		3.485 (270.7)	
31-35 (n=19)	39.66 (451.6)		3.485 (82.45)	
BMI	median (range)	p value	median (range)	p value
≤ 30 (n=30)	177.7 (1114)	0.4520	3.485 (609.4)	0.6803
> 30 (n=54)	39.66 (979.5)		3.485 (419.5)	
HSV-2	median (range)	p value	median (range)	p value
Indeterminate (n=18)	239.9 (946.6)	0.2029	3.485 (609.4)	0.0178
Negative (n=91)	204.2 (1114)		3.485 (419.5)	
Positive (n=56)	39.66 (26.4)		3.485 (250.1)	
Chlamydia Trachomatis	median (range)	p value	median (range)	p value
Negative (n=125)	79.31 (1114)	0.2209	3.485 (270.7)	0.3218
Positive (n=42)	251 (946.6)		3.485 (609.4)	
Neisseria gonorrhoeae	median (range)	p value	median (range)	p value
Negative (n=160)	165.2 (1114)	0.8045	3.485 (609.4)	0.4793
Positive (n=7)	39.66 (946.6)		3.485 (419.5)	
PSA	median (range)	p value	median (range)	p value
Negative (n=148)	177.7 (1114)	0.3106	3.485 (609.4)	0.6235
Positive (n=19)	39.66 (520.5)		3.485 (204.7)	

Abbreviations: BMI, body mass index; HSV, herpes simplex virus type 2; PSA, prostate specific antigen; TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted. *Indicates $p < 0.05$ by Mann Whitney U/Kruskal Wallis test after adjusting for multiple comparisons using a false discovery rate step-down procedure.

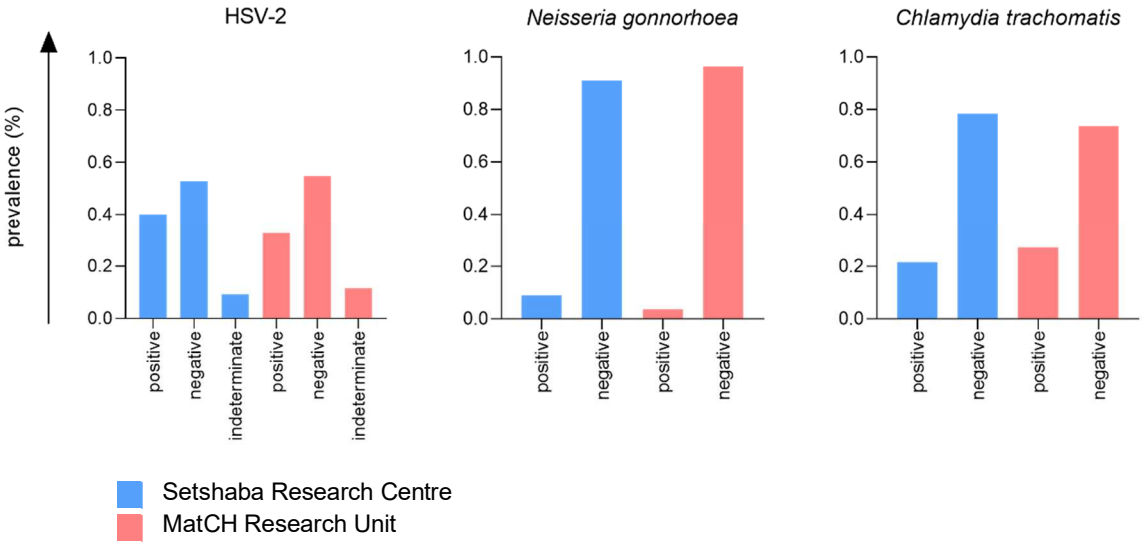

Figure 1. Sexually transmitted infection (STI) prevalence (%) across sites. Women from Setshaba Research Centre (n=55; blue) and MatCH Research unit (n=113; pink) were tested for the presence of HSV-2 antibodies in blood, and *N. gonorrhoea* and *C. trachomatis* in endocervical swabs using GeneXpert. Fisher's exact test was used for comparisons. Abbreviations: HSV-2, herpes simplex virus type 2.

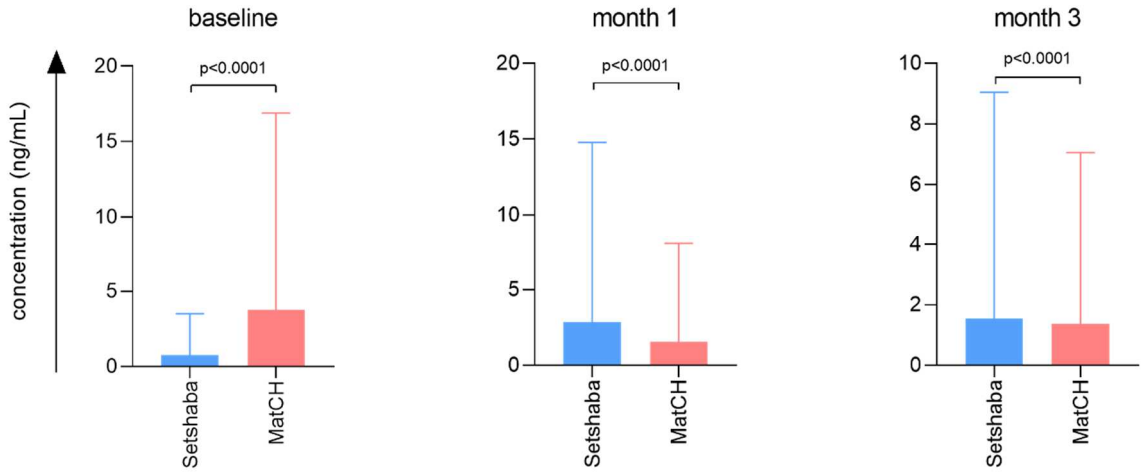

Figure 2. Prostate specific antigen (PSA) concentrations across sites. PSA levels were measured in lateral vaginal wall swab samples using ELISA. Mann Whitney U test was used to compare PSA concentrations between women from Setshaba Research Centre (n=55; blue) and MatCH Research Unit (n-113; pink). Abbreviations: PSA, Prostate specific antigen. P-values <0.05 were considered statistically significant.

Immune mediator detection

Generally, strong positive correlations were observed between duplicate immune mediator measurements (figure 3 A-K). Spearman's correlation coefficient between duplicate cytokine concentration readings was generally high, with all correlation coefficients >0.87, with the exception of MIP-1β (0.72). The percentage of samples with undetectable cytokine/SLPI concentrations ranged from <1 to 27% for most immune mediators (table 2, figure 4). The exceptions were MIP-1β and RANTES, which were undetectable in 42% and 73% of samples, respectively.

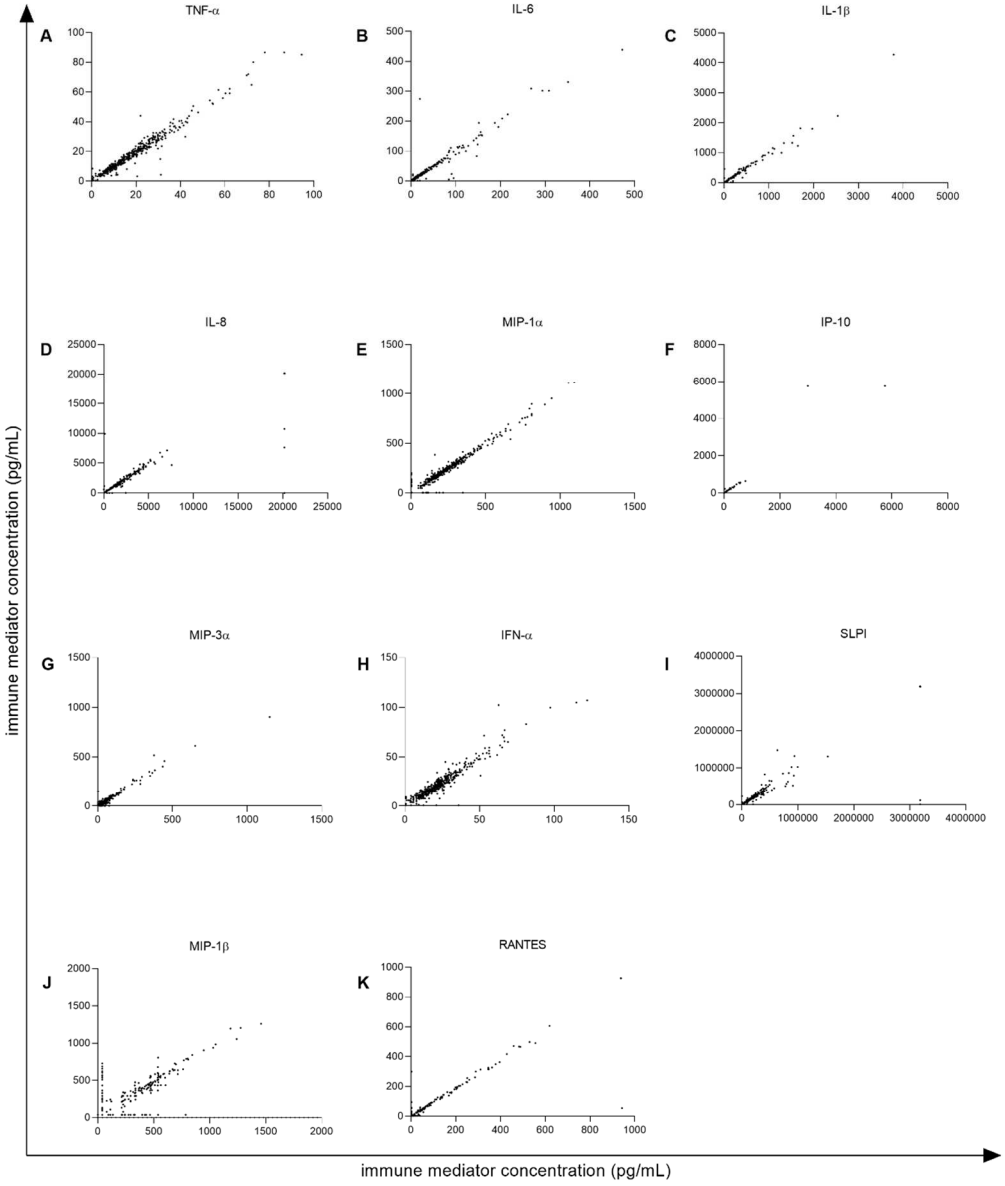

Figure 3 (A-K). Correlations between duplicate immune mediator concentrations (pg/mL). Genital immune mediator concentrations in lateral vaginal wall swabs were measured in duplicate using Luminex and duplicate values were plotted against each other in the scatter plots. Abbreviations: TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, Interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted.

Table 2. Correlation analysis of duplicate measurements per immune mediator.

Immune mediator	Spearman rho	p value	% Undetected
TNF-α	0.9665	<0.0001	0.034205231
IL-6	0.9334	<0.0001	0.004024145
IL-1β	0.9653	<0.0001	0.026156942
IL-8	0.9530	<0.0001	0.054325956
MIP-1α	0.9647	<0.0001	0.175050302
IP-10	0.9489	<0.0001	0.076458753
MIP-3α	0.8741	<0.0001	0.269617706
IFN-α	0.9303	<0.0001	0.008048290
SLPI	0.9761	<0.0001	0.028169014
MIP-1β	0.7246	<0.0001	0.416498994
RANTES	0.9141	<0.0001	0.730382294

Abbreviations: TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, Interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted.

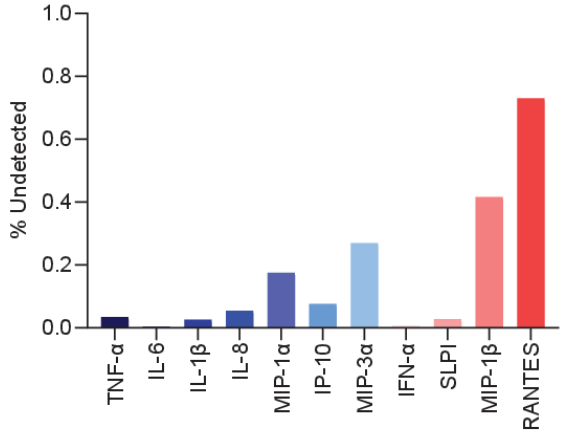

Figure 4. Percentage of samples with undetectable immune mediator concentrations. Genital immune mediator concentrations in lateral vaginal wall swabs were measured in duplicate using Luminex and the proportion of undetectable concentrations per immune mediator was determined. Abbreviations: TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, Interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted.

Analysis of absolute immune mediator concentrations

The copper IUD was associated with rapid increases in inflammatory markers following contraceptive initiation. Pro-inflammatory IL-6 (figure 5B) and IL-1β (figure 5C) and chemotactic IL-8 (figure 5D), MIP-1α (figure 5E), IP-10 (figure 5F), and MIP-1β (figure 5J) were significantly elevated one month following copper IUD insertion after adjusting for multiple comparisons. While RANTES was significantly elevated one-month following copper IUD insertion, this did not remain significant after adjusting for multiple comparisons (figure 5K). Concentrations of the pro-inflammatory IL-6 and chemotactic IP-10 remained significantly raised compared to baseline after three months of contraceptive use, although to a lesser extent when compared to month one and were not significant after adjusting for multiple comparisons. A slight decrease was observed in SLPI concentrations (figure 5I) one month post contraceptive initiation, however this was not statistically significant and concentrations of this protein at three months following contraceptive initiation were comparable to baseline. Insertion of copper IUD was not associated with changes in concentrations of TNF-α (figure 4A) and IFN-α (figure 5H).

Initiation of DMPA-IM was not associated with any significant changes in immune mediator concentrations. One-month following contraceptive initiation, slight decreases in TNF-α (figure 6A) and SLPI (figure 6I) concentrations were observed, however these changes were not significant. While concentrations of IFN-α (figure 6H) decreased three months after contraceptive initiation, this change was not statistically significant after adjusting for multiple comparisons.

No changes in immune mediator concentrations were evident one-month post LNG insertion, but at three months, concentrations of TNF-α (figure 7A), IP-10 (figure 7E), MIP-3α (figure 7G) and SLPI (figure 7I) were significantly raised relative to baseline. Concentrations of the chemokine IL-8 (figure 7D) were elevated at three months post contraceptive initiation but this association was not significant after adjusting for multiple comparisons.

Line graphs of this analysis visualizing changes in immune mediator concentrations in individual women are shown in the appendix (figure S3-S5). A contingency analysis revealed no significant differences in RANTES and MIP-1β at baseline (figure 8).

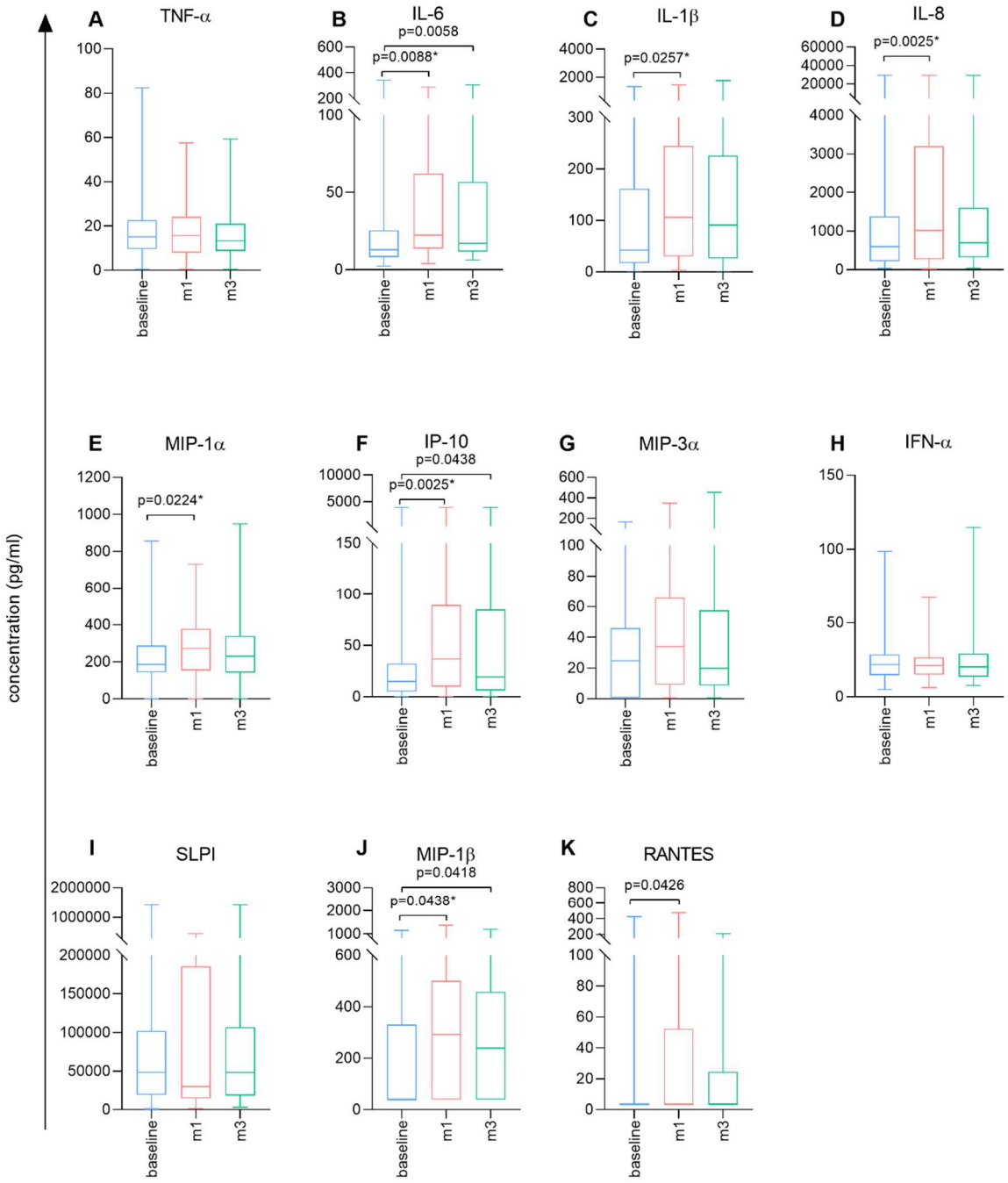

Figure 5 (A-K). Genital immune mediator concentrations (pg/mL) at baseline, one month following insertion of copper IUD (m1) and three months (m3) following insertion of copper IUD (n=55). Genital immune mediator concentrations in lateral vaginal wall swabs were measured using Luminex. Wilcoxon signed rank test was used for comparisons and p-values were adjusted for multiple comparisons using a false discovery rate step-down procedure. Abbreviations: m1, one-month post copper IUD insertion; m3, three months post copper IUD insertion; TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted. * Indicates $p < 0.05$ after adjusting for multiple comparisons.

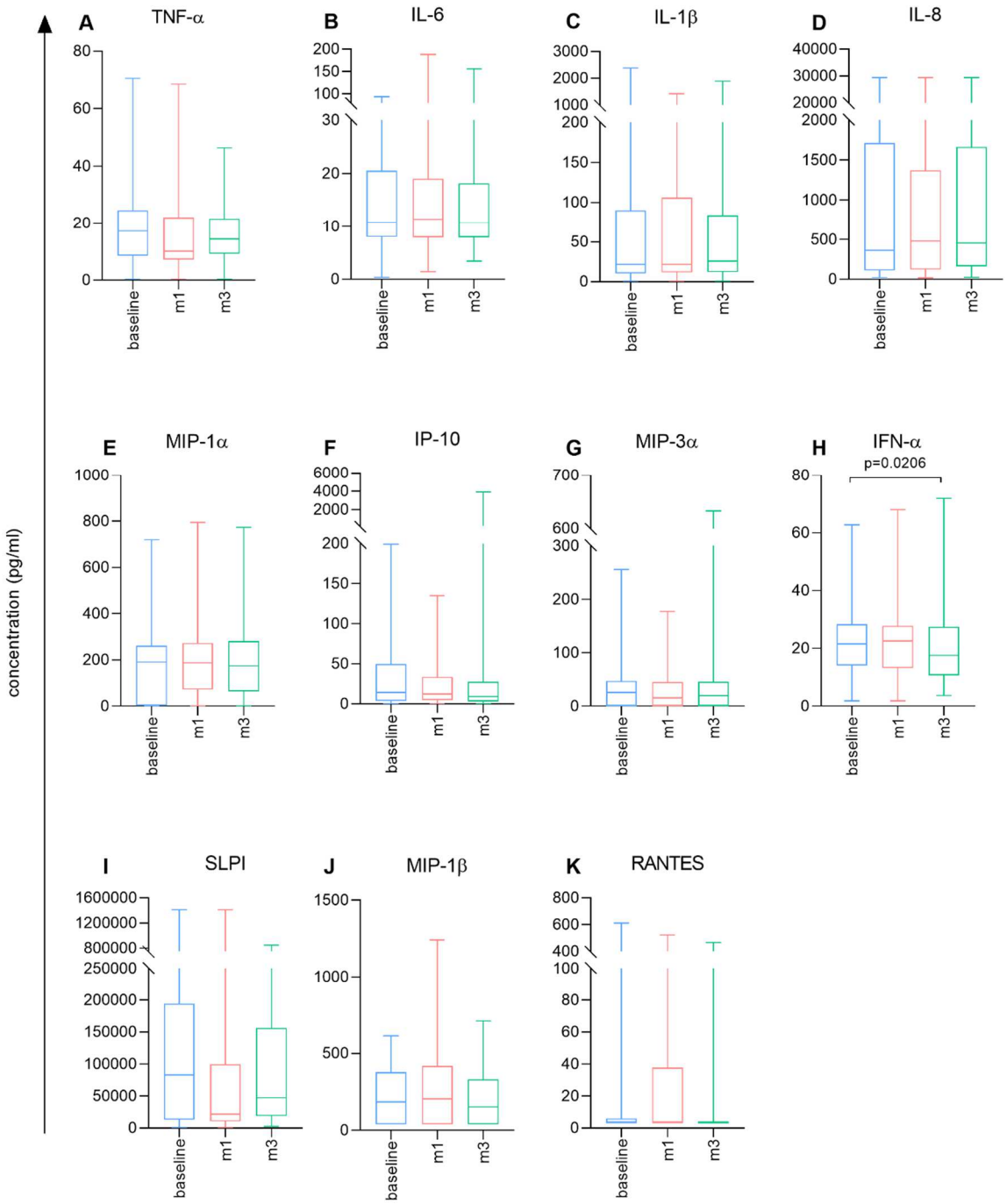

Figure 6 (A-K). Genital immune mediator concentrations (pg/mL) at baseline, one month following initiation of DMPA-IM (m1) and three months (m3) following initiation of DMPA-IM (n=53). Genital immune mediator concentrations in lateral vaginal wall swabs were measured using Luminex. Wilcoxon signed rank test was used for comparisons and p-values were adjusted for multiple comparisons using a false discovery rate step-down procedure. Abbreviations: m1, one-month post DMPA-IM initiation; m3, three months post DMPA-IM initiation; TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, Interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted. * Indicates $p < 0.05$ after adjusting for multiple comparisons.

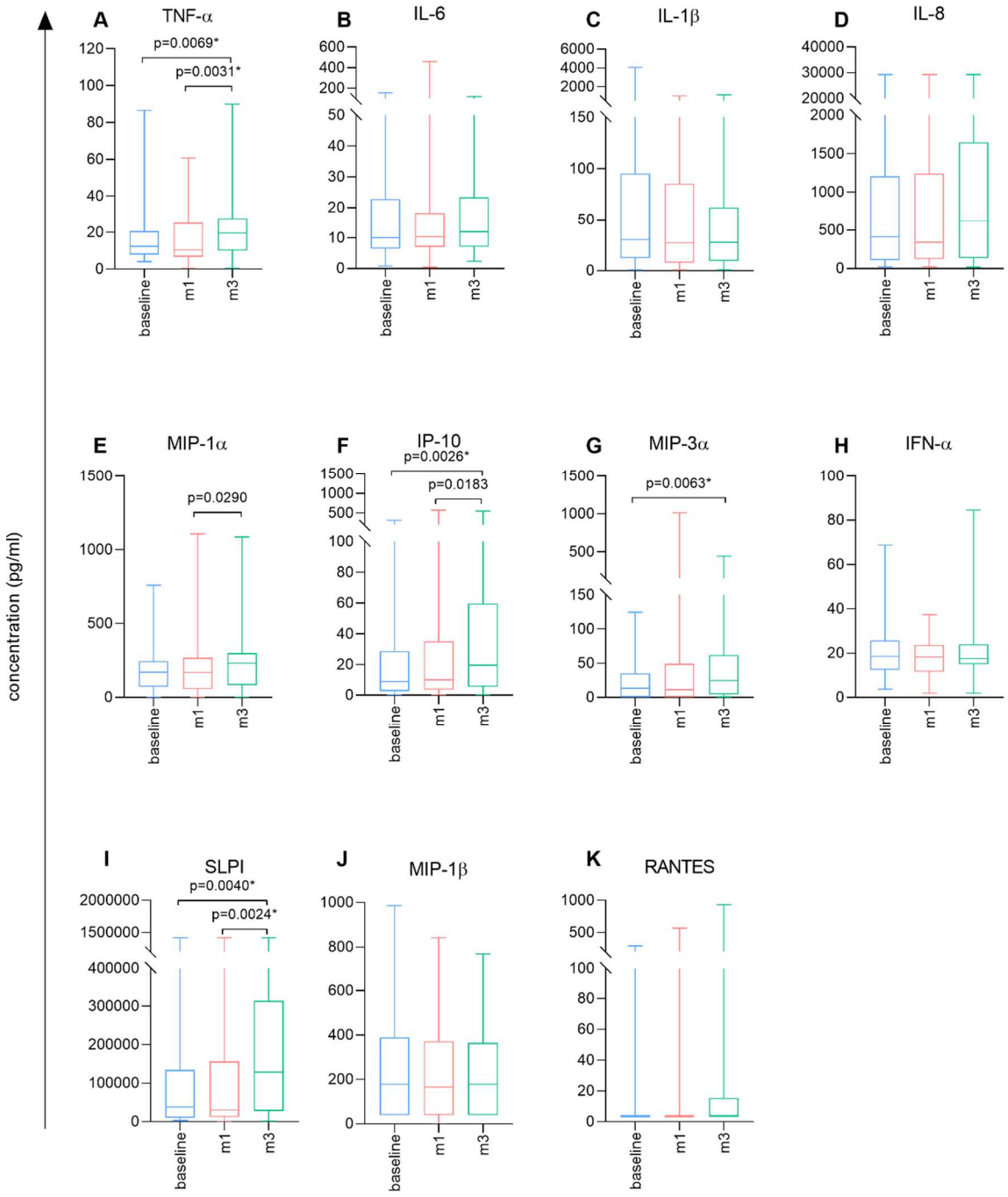

Figure 7 (A-K). Genital immune mediator concentrations (pg/mL) at baseline, one month following insertion of LNG Implant (m1) and three months (m3) following insertion of LNG Implant (n=59). Genital immune mediator concentrations in lateral vaginal wall swabs were measured using Luminex. Wilcoxon signed rank test was used for comparisons and p-values were adjusted for multiple comparisons using a false discovery rate step-down procedure. Abbreviations: m1, one-month post LNG insertion; m3, three months post LNG insertion; Cu-IUD, Copper intrauterine device; TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted. * Indicates $p < 0.05$ after adjusting for multiple comparisons.

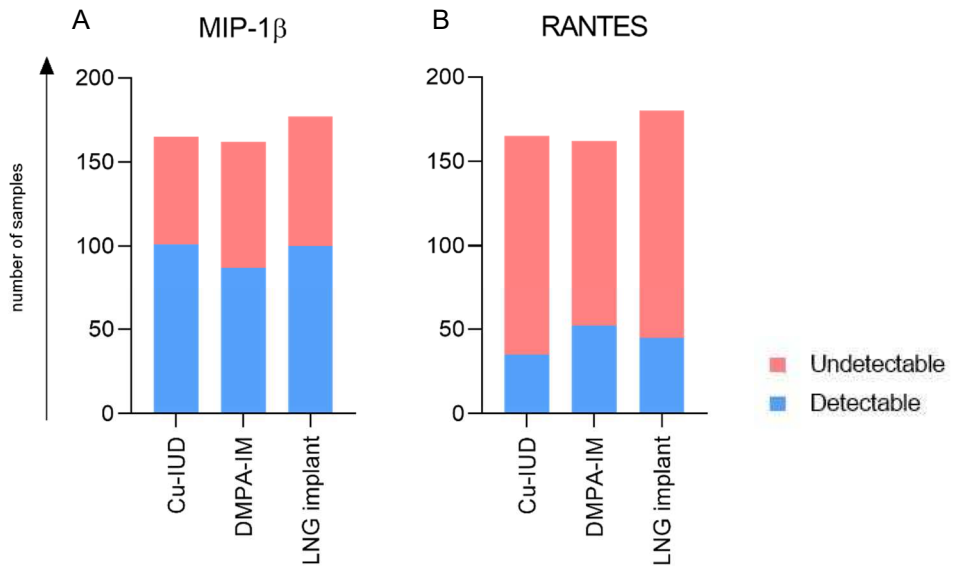

Figure 8. Contingency analysis of MIP-1β (A) and RANTES (B) by contraceptive arm. Genital immune mediator concentrations in lateral vaginal wall swabs were measured in duplicate at baseline. The concentrations for these two cytokines were grouped by arm, namely copper IUD (n=52), DMPA-IM (n=50) and LNG implant users (n=56) and categorized into detectable (blue) and undetectable (pink) concentrations. Fisher's exact test was used for comparisons. Abbreviations: Cu-IUD, copper intrauterine device; DMPA-IM, depomedroxyprogesterone acetate; LNG, levonorgestrel; MIP, macrophage inflammatory protein, RANTES, regulated on activation, normal T cell expressed and secreted.

Analysis of fold change in immune mediator concentrations

Fold changes (FC) in immune mediator concentrations were compared between the different contraceptive methods at one- and three-months following contraceptive initiation. One month post contraceptive initiation, fold change values were generally higher in copper IUD users when compared to DMPA-IM and the LNG implant. Increases in the concentrations of the pro-inflammatory cytokines TNF-α (1.2-fold, figure 9A), IL-6 (1.5-fold, figure 9B), IL-1β (1.8-fold, figure 9C), the chemokines IL-8 (1.4-fold, figure 9D), MIP-1α (1.2-fold, figure 9E), IP-10 (1.6-fold, figure 9F), MIP-3α (1.5-fold, figure 9G), SLPI (1.2-fold, figure 9I) as well as MIP-1β (1.3-fold, figure 9K) were observed. After adjusting for multiple comparisons this trend was statistically significantly higher for the chemokine MIP-1β (figure 9K) and the cytokine IL-1β (figure 9C) in the copper IUD arm when compared to the other contraceptive methods and the LNG implant, respectively. Slight to no changes in immune mediator concentrations were evident one-month post contraceptive initiation in DMPA-IM and LNG implant users, with the exception of a greater increases in concentrations of IL-1β (1.2-fold) in DMPA-IM users compared to women using the LNG implant (figure 9C) after adjusting for multiple comparisons. A non-significant 1.4-fold increase in IP-10 concentrations in LNG implant users was also observed (figure 9F). Additionally, with a fold change value below one, concentrations of TNF-α (0.9-fold, figure 9A) and SLPI (0.7-fold, figure 9I) were decreased in DMPA-IM users one month following injection with the contraceptive relative to baseline. No changes in RANTES (figure 9J) levels were evident one month post contraceptive initiation.

Three months following contraceptive initiation, concentrations of IL-8 (1.2- and 1.4-fold, figure 10D), MIP-1α (1.1- and 1.3-fold, figure 10E), IP-10 (1.5- and 1.6-fold, figure 10F), MIP-3α (1.2- and 1.2-fold, figure 10G) and SLPI (1.2- and 1.8-fold, figure 10I) were changed in women using both, the copper IUD and LNG implant, respectively. Compared to DMPA-IM users this change was significant for IL-6 in copper IUD users after adjusting for multiple comparisons. Of the two contraceptive methods, LNG implant insertion was generally associated with higher fold changes in these immune mediators at three months. Use of the implant was additionally associated with a 1.3-fold increase in TNF-α levels three months post contraceptive initiation that was significant compared to the DMPA-IM arm, but fell away after adjusting for multiple comparisons (figure 10A). A 1.9- and 1.6-fold change in IL-1β (figure 10C) and IL-6 (figure 10B), respectively, were observed at three months following copper IUD insertion, with the latter remaining significant compared to women using DMPA-IM after adjusting for multiple comparisons. While levels of IFN-α were lower three-months post

DMPA-IM injection (0.9-fold, figure 10H) relative to baseline, concentrations of IL-8 (figure 10D) and SLPI (figure 10I) were slightly elevated with 1.1- and 1.3-fold increases, respectively. However none of these changes were significant in the DMPA-IM arm compared to the copper IUD or LNG arms. No changes in RANTES (figure 10J) and MIP-1β (figure 10K) levels were evident three months post contraceptive initiation.

The cumulative median fold changes for all immune mediators were used to summarise and assess overall changes in genital cytokine profiles for each contraceptive method at one- and three-months post initiation (figure 11). Levels of immune mediators were raised in copper IUD users both one- (cumulative FC: 14.8) and three-months (cumulative FC: 13.8) following insertion. Additionally, LNG implant use was associated with an overall increase in genital immune mediator levels three months following insertion (cumulative FC: 13.8). No overall changes in cytokine profiles were observed in DMPA-IM users, with cumulative fold changes of ~11 for each time-point.

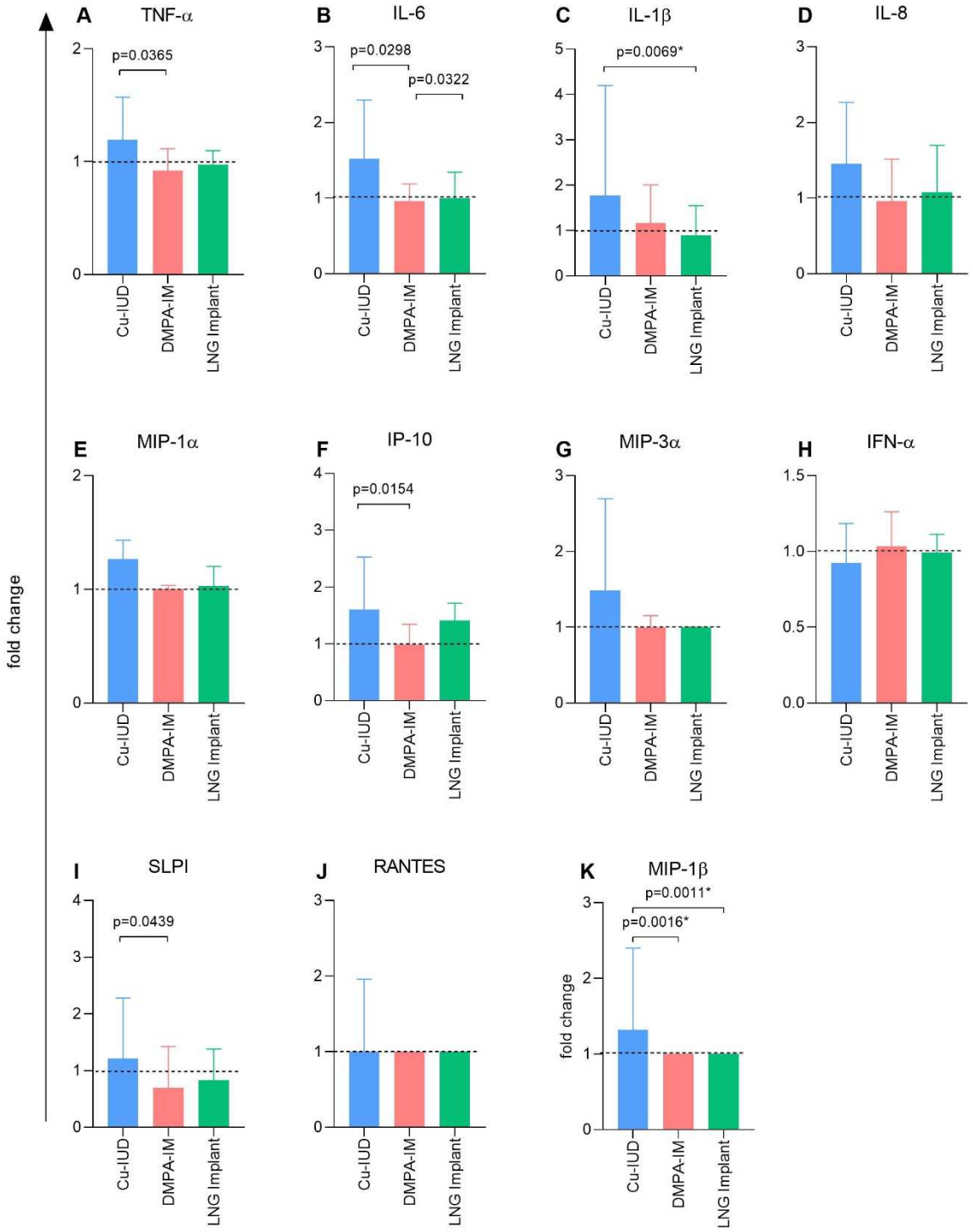

Figure 9 (A-K). Fold changes in genital immune mediator concentrations one month following initiation of copper IUD (n=52), DMPA-IM (n=50) or LNG implant (n=56) contraceptives. Genital immune mediator concentrations in lateral vaginal wall swabs were measured using Luminex. Mann-Whitney U test was used for comparisons and p-values were adjusted for multiple comparisons using a false discovery rate step-down procedure. Abbreviations: Cu-IUD, Copper intrauterine device; DMPA-IM, intramuscular depomedroxyprogesterone acetate; LNG, levonorgestrel; TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted. * Indicates $p < 0.05$ after adjusting for multiple comparisons. The dotted lines indicate a 1-fold change, i.e. no change in immune mediator concentration.

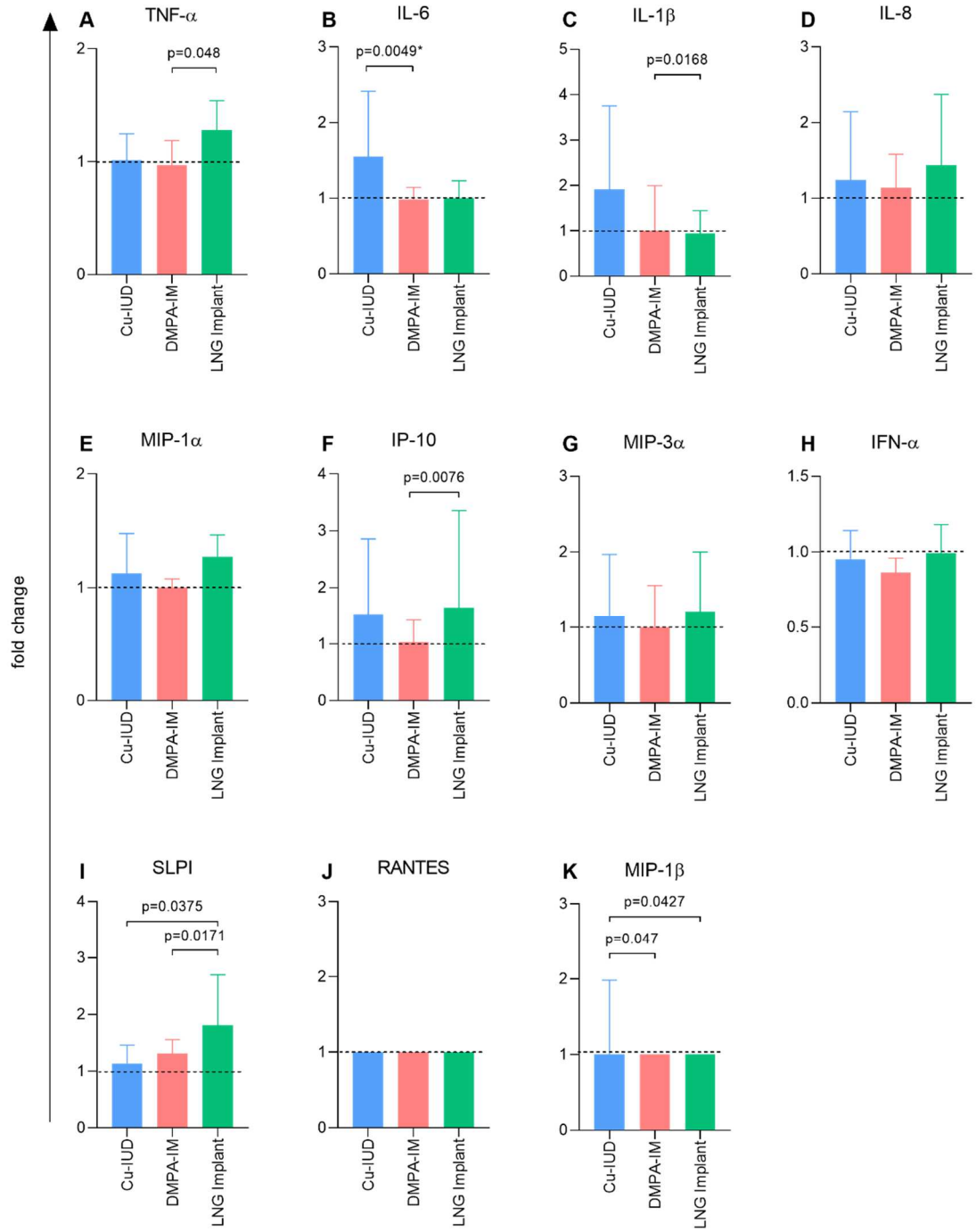

Figure 10 (A-K). Fold change of genital immune mediator concentrations three months following initiation of copper IUD (n=55), DMPA-IM (n=53) and LNG implant (n=59) contraceptives. Genital immune mediator concentrations in lateral vaginal wall swabs were measured using Luminex. Mann-Whitney U test was used for comparisons and p values were adjusted for multiple comparisons using a false discovery rate step-down procedure. Abbreviations: Cu-IUD, Copper intrauterine device; DMPA-IM, intramuscular depo-medroxyprogesterone acetate; LNG, levonorgestrel; TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted. * Indicates $p < 0.05$ after adjusting for multiple comparisons. The dotted lines indicate a 1-fold change, i.e. no change in immune mediator concentration.

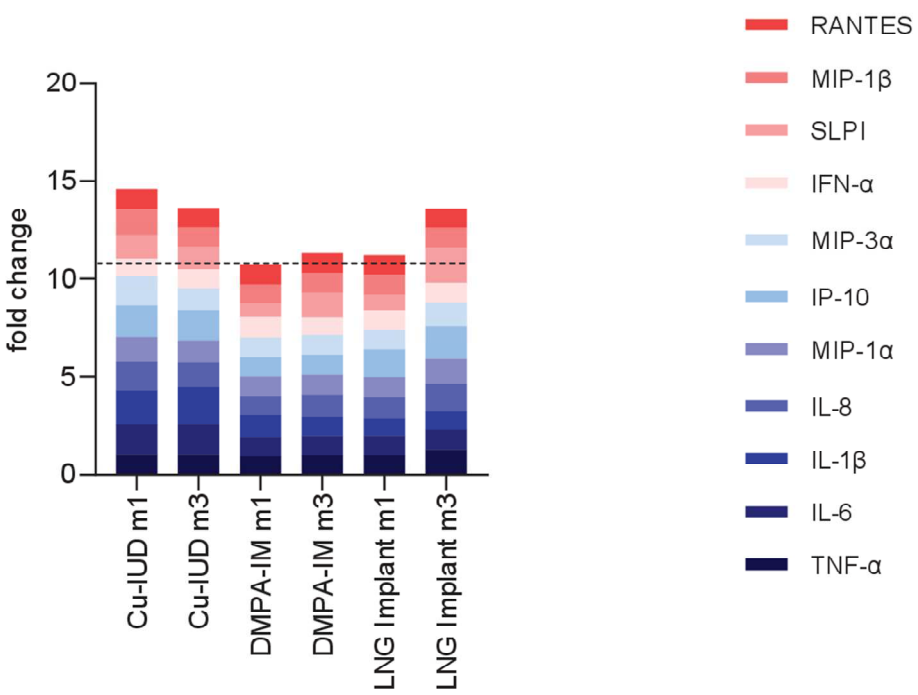

Figure 11. Cumulative median fold change values of immune mediators per contraceptive method one- and three- months post copper IUD insertion (n=55), DMPA-IM initiation (n=53) and LNG implant insertion (n=59). Genital immune mediator concentrations in lateral vaginal wall swabs were measured using Luminex. Median fold change values for each cytokine were stacked to assess overall changes in genital cytokine profiles. Abbreviations: Cu-IUD, Copper intrauterine device; DMPA-IM, intramuscular depo-medroxyprogesterone acetate; LNG, levonorgestrel; m1, one month post contraceptive initiation; m3, 3 months post contraceptive initiation; TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted. The dotted line indicates a cumulative 11-fold change, i.e. no change in immune mediator concentrations.

Principal component analysis

PCA was used to group pro-inflammatory cytokines, chemokines and all cytokines to generate estimates for each group. The magnitude of change (MOC) of these estimates was assessed one- and three months following contraceptive initiation. Differences in pro-inflammatory, chemokine and overall cytokine profiles between the methods of contraception were assessed using Mann-Whitney U test. Of the eleven immune mediators analysed in this study, three are classified as pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) and six are classified as chemotactic cytokines (IL-8, MIP-1α, IP-10, MIP-3α, MIP-1β and RANTES). The immune mediator IFN-α was also considered for the overall profile group in this analysis.

One-month post insertion, copper IUD use was associated with a significantly higher MOC in component estimates for pro-inflammatory cytokines (figure 12A), chemokines (figure 12B) as well as overall immune mediator profiles (figure 12C) when compared to DMPA-IM and LNG implant. While minimal changes in pro-inflammatory (figure 12A) as well as chemotactic

profiles (figure 12B) were observed at one month following DMPA-IM initiation, the trend was towards a decrease in pro-inflammatory cytokines, while chemokines tended to increase in concentration. The overall immune mediator profile tended to increase. LNG implant use was associated with a slight decrease in pro-inflammatory (figure 12A), as well as chemotactic cytokines (figure 12B), one month after contraceptive initiation. These changes were small but together resulted in a trend towards a decrease in overall immune mediator profile (figure 12C).

Similar to one-month following contraceptive initiation, copper IUD use was associated with raised pro-inflammatory (figure 13A), chemotactic (figure 13B) as well as overall immune mediator profiles (figure 13C) three months following contraceptive initiation. This change was smaller in magnitude compared to one-month post copper IUD insertion, but was statistically significant for pro-inflammatory cytokine and overall cytokine profiles compared to the DMPA arm. While the trend was towards a slight increase in chemokine (figure 13B) concentrations at three months following DMPA-IM initiation, pro-inflammatory cytokines tended to decrease (figure 13A) and this trend persisted towards a reduction in overall immune mediator profiles (figure 13C). Pro-inflammatory cytokine profiles tended to increase three months post LNG implant insertion (figure 13A), and this was accompanied by an increase in chemotactic cytokine profiles (figure 13B). Together this resulted in a substantial increase in overall immune mediator profiles (figure 13C), however none of the changes in the LNG arm were statistically significant compared to the other arms.

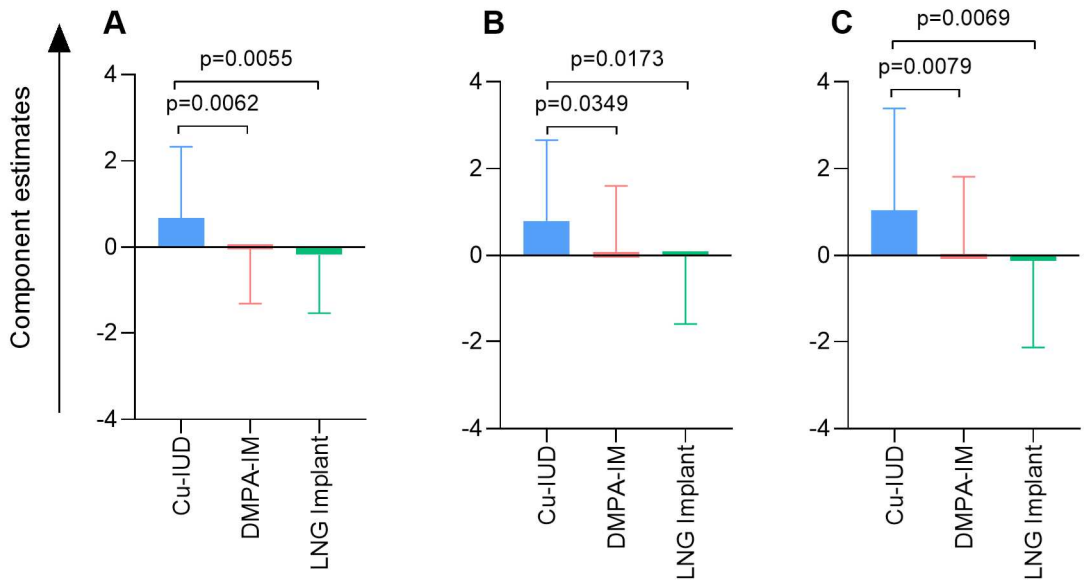

Figure 12. **Magnitude of change in principal component estimates for pro-inflammatory cytokine profiles (A), chemokine profiles (B) and overall cytokine profiles (C) between baseline and one month after contraceptive initiation.** Genital immune mediator concentrations in lateral vaginal wall swabs were measured using Luminex. Principal component analysis was used to group pro-inflammatory cytokines and chemokines and to generate estimates for each group. Baseline estimates were subtracted from month one estimates to generate the magnitude of change. Mann-Whitney U test was used for comparison of estimates between contraceptive arms. Abbreviations: Cu-IUD, Copper intrauterine device; DMPA-IM, intramuscular depo-medroxyprogesterone acetate; LNG, levonorgestrel. P<0.05 were considered statistically significant.

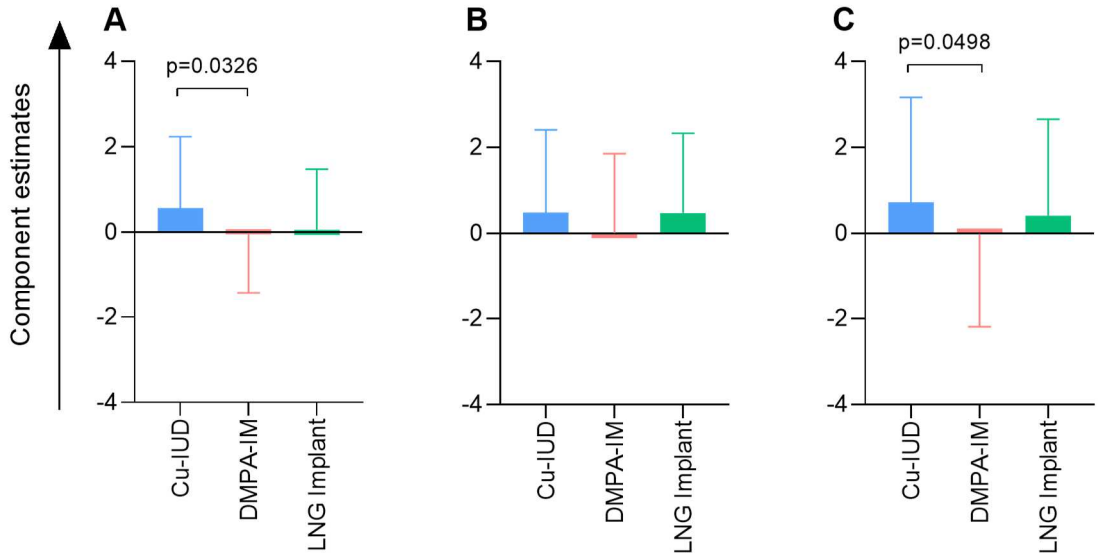

Figure 13. **Magnitude of change in principal component estimates for pro-inflammatory cytokine profiles (A), chemokine profiles (B) and overall cytokine profiles (C) between baseline and three months after contraceptive initiation.** Genital immune mediator concentrations in lateral vaginal wall swabs were measured using Luminex. Principal component analysis was used to group pro-inflammatory cytokines and chemokines and to generate estimates for each group. Baseline estimates were subtracted from month three estimates to generate the magnitude of change. Mann-Whitney U test was used for comparison of estimates between contraceptive arms. Abbreviations: Cu-IUD, Copper intrauterine device; DMPA-IM, intramuscular depo-medroxyprogesterone acetate; LNG, levonorgestrel. P<0.05 were considered statistically significant.

Exploratory PCA was performed using the log-transformed FC values of all pro-inflammatory and chemotactic cytokines, as well as IFN-α. One-month following contraceptive initiation, Principal component 1 (PC1) explained 40% of the variation in immune mediators while PC2 explained 14% (figure 14A). Immune mediators most strongly associated with PC1 at one month post contraceptive initiation included IL-8, IL-1β, MIP-3α, IL-6 and MIP-1α (figure 14C). While these loadings were all positive, RANTES, IL-8, MIP-1β, IL-1β and MIP-3α loaded negatively on PC2. Positive associations with PC2 at one month following contraceptive initiation were observed for TNF-α, IP-10, MIP-1α and IFN-α (figure 14D). Copper IUD users had higher scores on PC1 than DMPA-IM and LNG implant users one month following contraceptive initiation, resulting in the copper IUD cluster being shifted to the right compared to those of DMPA-IM and LNG users (figure 14A). This indicates that concentrations of the cytokines associated with PC1 tended to be higher in copper IUD users compared to those of DMPA-IM and LNG users.

Three months following contraceptive initiation, PC1 explained 42% of the variation in immune mediators, while PC2 explained 12% (figure 14B). The strongest positive associations between immune mediators and PC1 were observed for IL-8, IL-6, MIP-3α, IL-1β, IP-10 and MIP-1α (figure 14E). TNF-α, IFN-α, IP-10 and MIP-1β loaded negatively on PC2, while MIP-1β, IL-1β, RANTES, IL-8, IL-6 and MIP-3α were positively associated with PC2 (figure 14F). Similar to one month post contraceptive initiation, copper IUD use was associated with higher scores on PC1 relative to DMPA-IM use three months following insertion. Additionally, LNG implant users shifted to the right, indicating raised levels of immune mediators positively associated with PC1 (figure 14B). However, it is worth noting that changes in cytokine profiles associated with copper IUD and LNG insertion were relatively small.

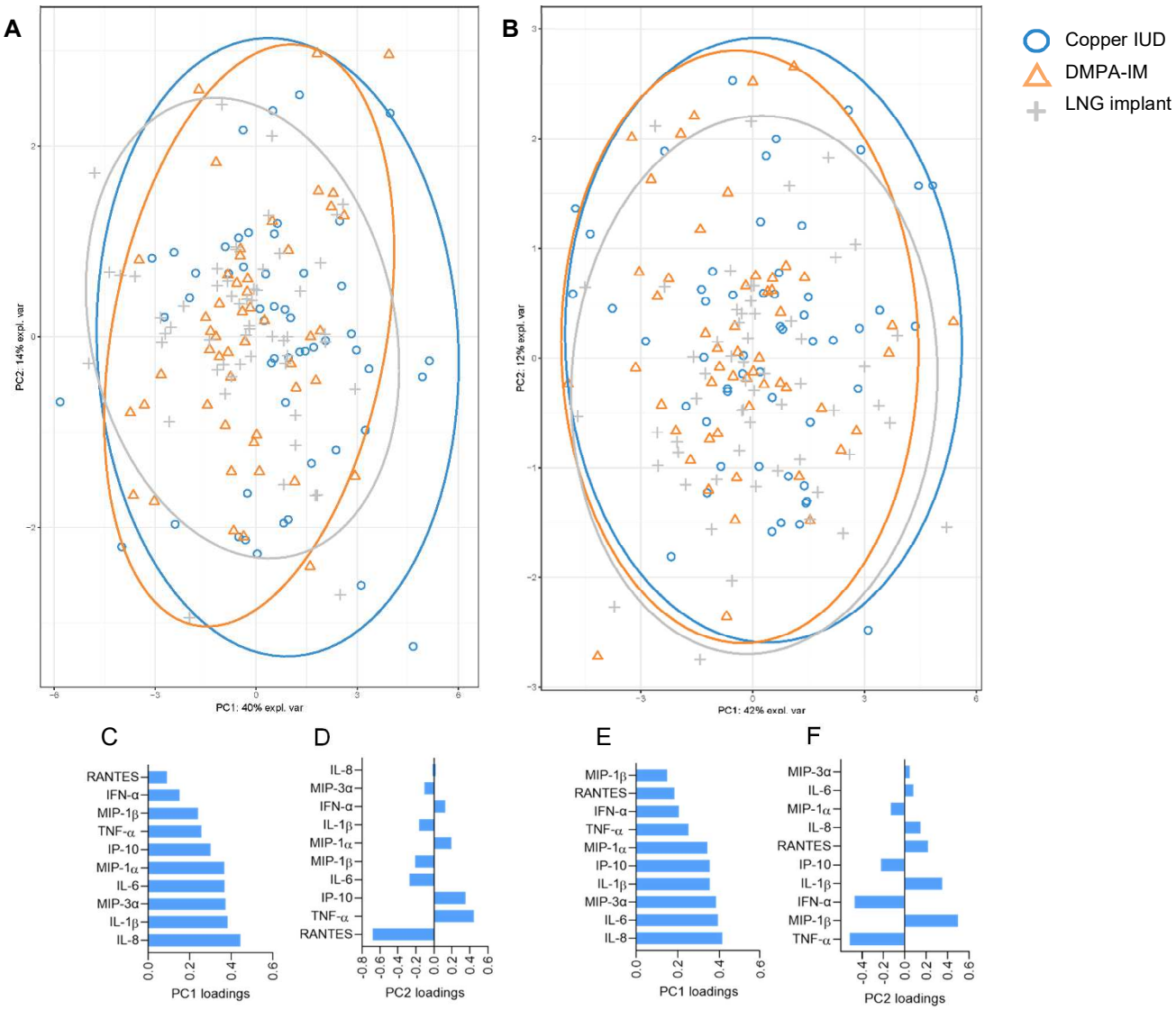

Figure 14. Principal component analysis (PCA) of log_{10}-transformed fold changes in immune mediator concentrations one- (A) and three- (B) months post contraceptive initiation. PCA was conducted using the R mixOmics package. Contraceptive methods are represented by colours and shapes, with copper IUD as blue circles, DMPA-IM as orange triangles and LNG implant as grey crosses. (C) The immune mediators most strongly associated with PC1 one month following contraceptive initiation included several chemokines (IL-8, MIP-3α and MIP-1α) as well as pro-inflammatory cytokines (IL-1β and IL-6). (D) The immune mediators that loaded positively on PC2 one month following contraceptive initiation included a pro-inflammatory cytokine (TNF-α) and chemotactic cytokines (IP-10 and MIP-1α) as well as IFN-α. Cytokines that were negatively associated with PC2 included RANTES, pro-inflammatory cytokines (IL-6 and IL-1β) as well as chemokines (MIP-1β and MIP-3α). (E) The immune mediators most strongly associated with PC1 three months following contraceptive initiation similarly included chemotactic cytokines (IL-8, MIP-3α, IP-10 and MIP-1α) and pro-inflammatory cytokines (IL-6 and IL-1β). (F) The immune mediators that loaded positively on PC2 included chemokines (MIP-1β and IL-8) and pro-inflammatory cytokines (IL-1β, IL-6). Cytokines that were negatively associated with PC2 included the pro-inflammatory TNF-α, IFN-α and the chemokines IP-10 and MIP-1α.

Figure 14 continued. Abbreviations: TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted.

Unsupervised hierarchical clustering

Unsupervised hierarchical clustering was used to visualize variation in inflammatory (TNF-α, IL-6 and IL-1β) and chemokine (IL-8, MIP-1α, IP-10, MIP-3α, MIP-1β and RANTES) concentrations in individual women and to cluster women according to the similarities in their immune mediator expression profiles. Figure 15 shows heat maps of the log_{10}-transformed FC values for each cytokine (columns) per individual (rows) one- (figure 15A) and three- months (figure 15B) post contraceptive initiation. The contraceptive method of each woman is indicated by a colour. The dendrogram above the heat map illustrates degrees of relatedness between genital cytokine profiles evident within the various women. While the length of horizontal branches has no meaning, vertical lines visualize the degree of cytokine relatedness, with longer branches indicating more distant relationships. The dendrogram on the left-hand side of the heat map indicates relationships between the expression profiles of the analysed cytokines across all women assessed in this study. Horizontal lines are a measure of the degree of similarity between the cytokine expression profiles of the individual women while vertical lines represent arbitrary lengths. At one-month following contraceptive initiation, the expression of IP-10, MIP-1α and TNF-α, as well as MIP-1β, MIP-3α, IL-6, IL-8 and IL-1β were more closely related, while the expression of RANTES proved to be more unique. The expression of the cytokines IP-10, MIP-1α and TNF-α as well as MIP-3α, IL-6, IL-8 and IL-1β remained closely related three months post contraceptive initiation. Additionally, RANTES and MIP-1β expression showed a high degree of relatedness.

Copper IUD use tended to be associated with higher levels of cytokines one- and three- months post contraceptive initiation (figure 15A, 15B). Women using DMPA-IM did not cluster together one month after DMPA-IM injection (figure 15A) but tended to cluster towards the bottom of the heat map three months following contraceptive initiation. While LNG implant use was associated with lower cytokine levels one month following insertion (figure 15A), a higher proportion of LNG implant users had elevated cytokine levels three months post contraceptive initiation (figure 15B).

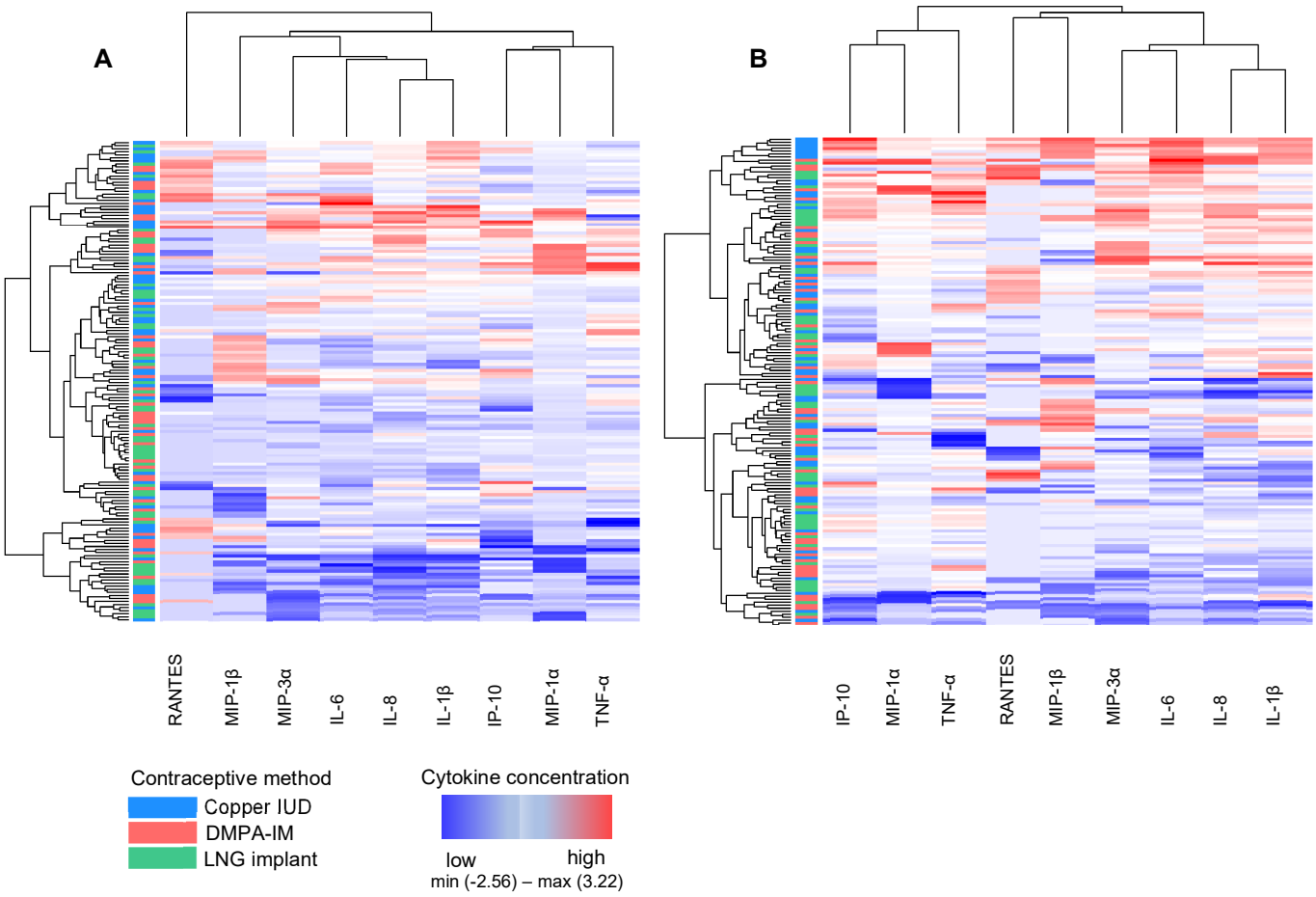

Figure 15. Unsupervised hierarchical clustering of women according to the similarities of their immune mediator expression profiles. Cytokine profiles were assessed in lateral vaginal wall swab samples from women using the copper IUD (n=55; blue blocks), DMPA-IM (n=53; orange blocks) and the LNG implant (n=59; green blocks) one month (A) and three months (B) following contraceptive initiation. Variations in cytokine fold changes are indicated using a colour scale that ranges from blue (low) through white to red (high). The dendrogram above the heat map illustrates degrees of relatedness between genital cytokine profiles. The dendrogram on the left-hand side of the heat map indicates relationships between the expression profiles of the analysed cytokines across all women assessed in this study. Abbreviations: TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; RANTES, regulated on activation, normal T cell expressed and secreted.

Longitudinal changes in immune mediators

Mixed effects linear regression analysis using log_{10}-transformed immune mediator data was performed to assess changes in immune mediator concentrations over time. Table 3 shows the analysis of copper IUD users relative to DMPA-IM users. A mixed effects linear regression analysis was selected to enable inclusion of participants with missing data. This approach allowed for analysis of longitudinal changes in cytokine measurements between arms, rather than comparison of absolute differences between individual time points. The β coefficient indicates the degree of change of an immune mediator in copper IUD users relative to DMPA-IM use, with a positive value showing a greater increase in cytokine concentration. Overall, concentrations of immune mediators tended to increase in copper IUD users relative to DMPA-IM users. The magnitude of change of all immune mediators was greater in copper IUD users between one month and baseline compared to DMPA-IM users, with the exception of IFN-α and MIP-1α which showed very little change. Similarly, the concentrations of most immune mediators tended to show a greater degree of change in copper IUD users three-months following contraceptive initiation when compared to DMPA-IM use, although the magnitude of change was small for TNF-α, IL-8, MIP-1 α, IFN-α, SLPI and RANTES. The biggest increase in immune mediator concentration in copper IUD users was observed for the chemotactic cytokine IP-10 with significant 0.404 and 0.384 log_{10} pg/ml increases one- and three- months after insertion relative to DMPA-IM users, respectively. The pro-inflammatory cytokine IL-6 and the chemokine MIP-1β also showed significantly greater increases in copper IUD compared to DMPA-IM users, both one- and three months following contraceptive initiation. A statistically significantly larger increase in TNF-α levels was observed in copper IUD compared to DMPA-IM users after one month of contraceptive use.

Results of the mixed effects linear regression analysis comparing LNG implant users to DMPA-IM users are presented in table 4. Although the changes in concentrations of IP-10 and SLPI tended to be greater in women using the LNG implant one month post contraceptive initiation when compared to DMPA-IM users, this was not statistically significant. The pro-inflammatory IL-1β and the chemotactic MIP-1α showed a non-significant trend towards a greater reduction in concentrations between month 1 and baseline in LNG implant users compared to women using DMPA-IM. While similar trends were observed at three months following contraceptive initiation, with TNF-α, IP-10, MIP-3α, SLPI and RANTES showing greater increases and IL-1β showing a smaller increase in LNG implant users compared to

women using DMPA-IM. Differences in the magnitude of change were only statistically significant for IP-10 and SLPI.

Table 3. Mixed effects linear regression analysis of changes in immune mediator concentrations in copper IUD relative to DMPA-IM users one- and three-months post contraceptive initiation.

Immune mediator	β coefficient	p value	95% Confidence Interval
TNF-α			
Month 1	0.198	0.037	0.012 - 0.383
Month 3	0.090	0.333	-0.092 - 0.272
IL-6			
Month 1	0.198	0.045	0.004 - 0.393
Month 3	0.214	0.028	0.024 - 0.405
IL-1β			
Month 1	0.226	0.149	-0.081 - 0.532
Month 3	0.217	0.158	-0.084 - 0.052
IL-8			
Month 1	0.183	0.251	-0.130 - 0.496
Month 3	-0.003	0.982	-0.311- 0.304
MIP-1α			
Month 1	0.040	0.824	-0.309 - 0.388
Month 3	-0.056	0.750	-0.400 - 0.287
IP-10			
Month 1	0.404	0.029	0.042 - 0.766
Month 3	0.384	0.034	0.029 - 0.739
MIP-3α			
Month 1	0.324	0.111	-0.074 - 0.722
Month 3	0.177	0.375	-0.214 - 0.569
IFN-α			
Month 1	-0.026	0.637	-0.134 - 0.082
Month 3	0.040	0.462	-0.066 - 0.146
SLPI			
Month 1	0.287	0.064	-0.016 - 0.591
Month 3	0.096	0.527	-0.202- 0.395
RANTES			
Month 1	0.215	0.149	-0.077 - 0.507
Month 3	0.092	0.532	-0.196 - 0.379
MIP-1β			
Month 1	0.241	0.026	0.029 - 0.453
Month 3	0.213	0.046	0.004 - 0.421

Shaded cells indicate significant p values ($p < 0.05$). Abbreviations: TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, Interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted.

Table 4. Mixed effects linear regression analysis of changes in immune mediator concentrations in LNG implant relative to DMPA-IM users one- and three-months post contraceptive initiation.

Immune mediator	β coefficient	p value	95% Confidence Interval
TNF-α			
Month 1	0.059	0.501	-0.110 - 0.225
Month 3	0.135	0.110	-0.030 - 0.300
IL-6			
Month 1	-0.046	0.625	-0.232 - 0.139
Month 3	0.053	0.571	-0.130 - 0.236
IL-1β			
Month 1	-0.151	0.281	-0.425- 0.123
Month 3	-0.156	0.258	-0.425 - 0.114
IL-8			
Month 1	-0.024	0.880	-0.338 - 0.290
Month 3	-0.044	0.781	-0.354 - 0.266
MIP-1α			
Month 1	-0.192	0.275	-0.535 - 0.152
Month 3	-0.001	0.995	-0.341 - 0.339
IP-10			
Month 1	0.143	0.347	-0.156- 0.443
Month 3	0.4401	0.003	0.146 - 0.735
MIP-3α			
Month 1	0.032	0.865	-0.341 - 0.406
Month 3	0.327	0.081	-0.041 - 0 .695
IFN-α			
Month 1	-0.042	0.431	-0.145 - 0.062
Month 3	0.039	0.449	-0.062- 0.141
SLPI			
Month 1	0.192	0.202	-0.103 - 0.487
Month 3	0.370	0.013	0.079 - 0 .660
RANTES			
Month 1	0.041	0.775	-0.240 - 0.322
Month 3	0.159	0.260	-0.118 - 0.437
MIP-1β			
Month 1	-0.031	0.754	-0.2258 - 0.163
Month 3	0.002	0.985	-0.189 - 0.193

Shaded cells indicate significant p values ($p < 0.05$). Abbreviations: TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, Interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted.

Changes in cytokine concentrations after adjustment for potential confounders

Multinomial logistic regression analysis using log_{10}-transformed FC in immune mediator concentrations was performed to compare changes in immune mediator concentrations in copper IUD and LNG implant users relative to DMPA-IM use, after adjusting for possible confounders, including site, age and infection status with chlamydia, gonorrhoea and HSV-2. PSA presence was not controlled for in this model, as it was not identified as a potential confounder in the baseline analysis. Multinomial logistic regression is used to predict categorical placement in a dependent variable based on multiple independent variables [118]. For example, with reference to the data presented in table 5, if the log_{10}-transformed FC of IP-10 increased by 1 unit at one month following copper IUD insertion, the multinomial log-odds ratio for being a copper IUD user, relative to the log-odds ratio of being a DMPA-IM user, would be expected to increase by 0.623 units while holding all other variables in the model constant (table 5). Significant increases associated with copper IUD compared to DMPA-IM use were observed for the pro-inflammatory cytokine IL-6 and the chemokine IP-10 at one- and three- months following contraceptive initiation after controlling for site, age and infection status with chlamydia, gonorrhoea and HSV-2 (table 5). LNG implant use, relative to DMPA-IM use, was associated with higher levels of IP-10 and SLPI three months following contraceptive initiation (table 6) after controlling for possible confounders.

Table 5. Multinomial logistic regression analysis of changes in immune mediator concentrations in copper IUD compared to DMPA-IM users one- and three- months following contraceptive initiation.

Immune mediator	β coefficient	p value	95% Confidence Interval	p value adjusted for confounders
TNF-α				
Month 1	0.962	0.035	0.068 - 1.856	0.065
Month 3	0.467	0.284	-0.387 - 1.321	0.405
IL-6				
Month 1	0.787	0.062	-0.038 - 1.613	0.041
Month 3	0.804	0.039	0.041 - 1.566	0.021
IL-1β				
Month 1	0.421	0.109	-0.095 - 0.937	0.094
Month 3	0.350	0.157	-0.135 - 0.836	0.093
IL-8				
Month 1	0.705	0.289	-0.239 - 0.804	0.338
Month 3	-0.011	0.960	-0.450 - 0.428	0.913
MIP-1α				
Month 1	0.059	0.793	-0.378 - 0.495	0.865
Month 3	-0.063	0.782	-0.508 - 0.382	0.965
IP-10				
Month 1	0.623	0.018	0.105 - 1.140	0.038
Month 3	0.478	0.031	0.045 - 0.912	0.029
MIP-3α				
Month 1	0.365	0.113	-0.087 - 0.817	0.162
Month 3	0.159	0.400	-0.212 - 0.530	0.368
IFN-α				
Month 1	-0.531	0.501	-2.078 - 1.016	0.524
Month 3	0.522	0.462	-0.870 - 1.914	0.385
SLPI				
Month 1	0.500	0.054	-0.009 - 1.010	0.111
Month 3	0.181	0.471	-0.313 - 0.675	0.548
RANTES				
Month 1	0.417	0.144	-0.142 - 0.977	0.100
Month 3	0.137	0.581	-0.351 - 0.625	0.509
MIP-1β				
Month 1	0.743	0.041	0.031 - 1.454	0.051
Month 3	0.728	0.044	0.020 - 1.436	0.059

The confounders included in this model were site, age and infection status with chlamydia, gonorrhoea and HSV-2. Shaded cells indicate significant p values ($p < 0.05$). Abbreviations: HSV-2; herpes simplex virus type 2, TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, Interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted.

Table 6. Multinomial logistic regression analysis of changes in immune mediator concentrations in LNG implant compared to DMPA-IM users one- and three- months following contraceptive initiation.

Immune mediator	β coefficient	p value	95% Confidence Interval	p value adjusted for confounders
TNF-α				
Month 1	0.245	0.552	-0.562- 1.051	0.804
Month 3	0.705	0.110	-0.160 - 1.569	0.142
IL-6				
Month 1	-0.248	0.560	-1.080 - 0.585	0.675
Month 3	0.216	0.577	-0.544 - 0.976	0.313
IL-1β				
Month 1	-0.227	0.369	-0.724 - 0.269	0.362
Month 3	-0.257	0.281	-0.724 - 0.210	0.396
IL-8				
Month 1	-0.115	0.651	-0.616 - 0.385	0.539
Month 3	-0.062	0.778	-0.495 - 0.371	0.853
MIP-1α				
Month 1	-0.244	0.271	-0.679 - 0.191	0.152
Month 3	0.011	0.962	-0.430 - 0.452	0.908
IP-10				
Month 1	0.190	0.438	-0.289 -0 .669	0.763
Month 3	0.562	0.011	0.127 - 0.996	0.012
MIP-3α				
Month 1	-0.018	0.936	-0.448 - 0.412	0.724
Month 3	0.309	0.102	-0.062 - 0.679	0.076
IFN-α				
Month 1	-0.776	0.316	-2.294 - 0.742	0.375
Month 3	0.513	0.464	-0.861 - 1.888	0.328
SLPI				
Month 1	0.301	0.228	-0.188 - 0.790	0.417
Month 3	0.661	0.012	0.147 - 1.174	0.012
RANTES				
Month 1	0.071	0.800	-0.478 - 0.620	0.571
Month 3	0.264	0.281	-0.216- 0.744	0.228
MIP-1β				
Month 1	-0.134	0.697	-0.810 - 0.541	0.706
Month 3	0.012	0.972	-0.679 - 0.704	0.969

The confounders included in this model were site, age and infection status with chlamydia, gonorrhoea and HSV-2. Shaded cells indicate significant p values ($p < 0.05$). Abbreviations: HSV-2; herpes simplex virus type 2; TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, Interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted.

Discussion

Immune mediator profiles in the FGT are thought to play an important role in the HIV risk of women. The effects of long-acting contraceptives, including DMPA-IM, the copper IUD and the LNG implant, on these cytokine profiles (and by extension HIV risk) remain largely unknown. The focus of this study was to investigate changes in immune mediator profiles associated with the initiation of DMPA-IM and compare these to LNG implant and copper IUD insertion. Furthermore, baseline demographic and biological factors that may alter immune mediator responses to these methods of contraception were evaluated. Correlates of immune mediator concentrations at baseline included clinical site, age and HSV-2 seropositivity. The copper IUD and LNG implant were associated with rapid increases in inflammatory markers following contraceptive initiation, while no significant changes were detected following DMPA-IM initiation. After adjusting for potential confounders, including site, age and infection status with chlamydia, gonorrhoea and HSV-2, IL-6 and IP-10 were significantly elevated in the copper IUD compared to the DMPA-IM arm at months 1 and 3, while IP-10 and SLPI were higher in the LNG implant arm at month 3 compared to the DMPA-IM arm.

Quality of mediator data

Generally, strong positive correlations were observed between duplicate immune mediator measurements. However, a high proportion of MIP-1β and RANTES concentrations were undetectable. Analysis of the standard curves generated during Luminex revealed that the lower detection limit was likely too high to reliably identify lower concentrations of these cytokines. Additionally, there is little consensus on the best method of mucosal sampling[119]. It has previously been suggested that endocervical swabs and cervicovaginal lavage (CVL) samples are optimal for maximum recovery of mediators in the FGT[120]. This could explain the low levels of MIP-1β and RANTES observed in this study, where lateral vaginal wall swabs were used. While the two cytokines were included in the downstream analysis, results should be interpreted with caution. The remaining immune mediators assessed in this study showed high correlation between duplicate values and detection levels were above 70%.

Baseline correlates of FGT immune mediator profiles

In this study, it was found that concentrations of the pro-inflammatory cytokine IL-6 and the chemokine MIP-3α were significantly higher in women from SRC, located in Pretoria, when compared to those from MRU, Durban. Previous studies in women from different locations within South Africa identified significant differences in the vaginal microbiome and hence inflammation levels. Lennard, *et al*. found that the prevalence of STIs was significantly higher

in women from Cape Town compared to those from Johannesburg[6]. The prevalence of *C. trachomatis*, in particular, was determined to be 3-fold higher in women from Cape Town. Although no differences in the prevalence of the STIs that were assessed in this study were found between the two sites, it is possible that the prevalence of other STIs, e.g. *Trichomonas vaginalis*, or BV differed. Another possibility is that there were slight differences in sampling methods between the two sites.

PSA is frequently measured as a biomarker of recent seminal plasma exposure[121]. The presence of this protein was not associated with significant differences in immune mediator concentrations, but the pro-inflammatory IL-6 and the chemotactic cytokine MIP-3α were slightly raised in its presence. While baseline PSA levels were significantly higher in women from the MRU, Durban, women from SRC showed higher concentrations of the protein at both follow up visits. Thus, women enrolled at SRC demonstrated more frequent unprotected sexual activity close to their study visits overall. A study of healthy Belgian women recently reported on a strong correlation between PSA and IL-6[122]. In addition, it has been shown that seminal plasma stimulates the production of several pro-inflammatory and chemotactic cytokines in the FGT, including IL-8, MCP-1 and GM-SCF in cervical cells lines[34]. The lack of significant associations between PSA and cytokines in this study may be due to the relatively small number of women who had detectable PSA in their FGT samples. However, based on this data, it seems that differences in sexual activity did not explain the differences in cytokine concentrations that were observed between study sites.

Women in the age groups of 18-20 and 21-24 years had elevated concentrations of pro-inflammatory IL-1β and the chemokines IL-8 and MIP-1α after adjusting for multiple comparisons. No significant differences in age was found between the two sites (figure S9). Importantly, an association between cytokine levels and age has been demonstrated previously in South African women, with younger women displaying higher inflammatory cytokine concentrations[4]. The study performed by Masson, *et al.* showed that elevated IL-6, IP-10, MCP-1 and MIP-1β concentrations were significantly associated with younger age. These results overlap with the association of young age and elevated immune mediator levels observed in this study. It has been suggested that, compared to adult women, the adolescent FGT is in a naïve state and immune responses to exogenous factors could be heightened in young women. While the role of HC use in women of different ages remains to be explored in the context of genital inflammation, studies have examined semen as an exogenous factor[123]. Generally, the immune system must be able to distinguish between semi-allogeneic semen from

sexual partners and potential pathogens for reproduction to be possible[124]. While semen should be tolerated, an inflammatory response must be mounted against pathogens. However, studies have shown that increased inflammation is observed in the genital tracts of women following semen exposure after a period of abstinence compared to women who were not abstinent[34]. Similarly, it is now hypothesized that the naïve state of the adolescent genital tract results in a lack of tolerance towards sex and semen, and exposure to these may result in a greater inflammatory response than seen in adult women[125]. Although it would be interesting to examine interactions between semen exposure and age that may influence cytokine concentrations, the relatively small number of PSA positive women in this study limited the power to conduct this analysis.

Women who tested positive for HSV-2 tended to have lower concentrations of immune mediators at baseline. All immune mediators, with the exception of MIP-1β, were present at lower concentrations in HSV-2 seropositive women, however, this trend was only statistically significant for the chemokine MIP-1α after adjusting for multiple comparisons. This is surprising given that previous studies have found that HIV target cell concentrations at the mucosa are increased during HSV-2 infection, regardless of the presence of lesions[39] and that subclinical levels of inflammation in the genital mucosa can persist for months after lesion resolution[40]. Infection status with *C. trachomatis* had a varied effect on immune mediator concentrations with some, including IL-1β, IL-8, MIP-1α, IP-10 and MIP-1β slightly raised in women who tested positive for the pathogen. Others, namely TNF-α, IL-6, MIP-3α, IFN-α and SLPI, were detected at lower levels in infected women, however, none of these associations remained significant after adjusting for multiple comparisons. Immune mediator concentrations were generally higher in women infected with *N. gonorrhoeae*, particularly, IL-6 and IL-8, however, this trend was also not statistically significant after adjusting for multiple comparisons. Previously, Masson *et al*. showed that women with the above STIs had elevated concentrations of several pro-inflammatory and chemotactic cytokines compared to women with no infections. Women with chlamydia, gonorrhoea or shedding HSV-2 had upregulated concentrations of cytokines that mediate chemotaxis of several immune cells, including T cells, natural killer cells, phagocytes, monocytes, dendritic cells, neutrophils, eosinophils, granulocytes and endothelial cells. Furthermore, cytokines involved in cellular apoptosis, viability, activation, proliferation and differentiation were upregulated in women with chlamydia, gonorrhoea and shedding HSV-2. The study did not however identify any associations with HSV-2 seropositivity[36]. Chlamydia infection has shown a strong association

with elevated levels of cytokines in numerous studies[36,126–128]; and while no significant associations were detected in the present study, the concentrations of several chemotactic cytokines tended to be elevated in women who tested positive for the pathogen. Interestingly, Barnabas *et al*. recently reported that *C. trachomatis* infection was associated with higher genital inflammation in women from Johannesburg compared to women from Cape Town[129]. This was due to clade differences – some clades of *C. trachomatis* proved to be more inflammatory than others. In this study, no difference in immune mediator levels was detected between *C. trachomatis* positive women from Durban compared to Pretoria (figure S8). It has been shown that infection of cervical and vaginal cell lines with *N. gonorrhoeae* resulted in upregulated IL-1, IL-6 and IL-8 production[130]. The cytokines IL-6 and IL-8 are under the transcriptional control by NF-κB and, while the former is a powerful chemoattractant and activator of neutrophils [131], the latter is considered one of the most potent cytokines in promoting inflammatory events through recruitment and activation of T cells and differentiation of B cells[132]. Although chlamydia and HSV-2 seropositivity were prevalent in this cohort, very few women had gonorrhoea (n=7), limiting the statistical power of this analysis. Mucosal cytokine production forms a central part of the immune response to pathogens in the vaginal environment and future studies to investigate broader immune pathways that drive these cytokine responses could provide crucial insight into the impact of STIs on the genital milieu[133,134].

Changes in FGT immune mediator concentrations associated with contraceptive use

While the benefits of HCs are immense, their role in HIV acquisition risk has long been discussed. Although the recent ECHO trial did not demonstrate statistically significant differences of HIV infection between women using DMPA-IM compared to those using the copper IUD or the LNG implant, the study was only powered to detect an increased risk above 50% and it furthermore remains unknown whether these forms of HCs increase HIV risk relative to other forms of contraception or no contraception[78]. Evaluation of the changes in the profiles of immune mediators that have previously been linked to HIV acquisition risk may provide further insight into the possibility of increased risk associated with these contraceptives.

Copper IUD

In this study, copper IUD use was associated with rapid increases of inflammatory markers following contraceptive initiation. Pro-inflammatory IL-6 and IL-1β and chemotactic IL-8, MIP-1α, IP-10, and MIP-1β were significantly elevated one month following copper IUD

insertion. Although to a lesser extent, concentrations of the pro-inflammatory IL-6 and chemotactic IP-10 remained significantly elevated after three months following copper IUD insertion. In line with this, using PCA estimates, it was found that overall pro-inflammatory, as well as chemotactic cytokine, profiles were significantly raised in copper IUD users compared to DMPA-IM and LNG implant users, particularly one-month post contraceptive initiation. In an exploratory PCA analysis, copper IUD users had higher scores on PC1, (onto which IL-8, IL-1β, MIP-3α, IL-6 and MIP-1α loaded positively), than DMPA-IM and LNG implant users one month following contraceptive initiation. This trend persisted until three months post contraceptive initiation. Unsupervised hierarchical clustering analysis further substantiated this observation as copper IUD use was again found to be associated with higher levels of cytokines one- and three- months post contraceptive initiation.

Importantly, a number of cytokines that were found to be present at higher concentrations following copper IUD use in this study have been identified as some of the most critical cytokines associated with HIV infection risk[4]. These include IL-8, MIP-1α and IP-10. IP-10, MIP-1α and MIP-1β are chemotactic for potential HIV target cells, including T cells, monocytes, macrophages, and dendritic cells[9,135,136]. Additionally, MIP-1α and MIP-1β bind directly to the CCR5 and therefore specifically target CCR5+ cells for recruitment to the genital mucosa[22]. The coreceptor plays a critical role in HIV transmission infection in the context of sexual transmission as infection is established exclusively by R5-tropic viruses[23]. Elevated levels of these cytokines are therefore proposed to increase recruitment of HIV target cells to the genital mucosa, in turn possibly increasing the likelihood of infection. The cytokines IL-6 and IL-8 play a vital role in initiating the inflammatory response and regulate the host defence against pathogens, mediating the innate immune response. Evidence has suggested that dysregulation of systemic IL-6 production is a major contributor to the pathogenesis of chronic inflammation[137]. In the context of HIV transmission, it has been demonstrated that high concentrations of IL-6 increase HIV expression *in vivo*[60]. Furthermore, HIV shedding was heightened in the presence of elevated IL-6 levels in cervicovaginal lavage obtained from HIV infected women[58]. The chemotactic cytokine IL-8 has also been associated with up to an 8-fold increase in HIV transmission in cervical explant tissue[61,138]. In addition, pro-inflammatory cytokines promote the expression of nuclear factor (NF)-κB. This influential transcription factor, in turn, directly binds to the HIV promoter and enhances its transcriptional capacity, thereby promoting productive infection further[24].

Although there is a lack of available data, a recent pilot study assessed changes in cervicovaginal cytokine levels following the insertion of different intrauterine devices. The authors reported that copper IUD insertion induced a typical mucosal inflammatory cytokine response one month following insertion, characterised by significant increases in IL-1α, IL-1β, IL-6 and TNF-α[139]. Since the copper IUD is a non-hormonal method of contraception, the possible mechanisms behind increased genital inflammation are relatively unexplored. Older studies have reported increased cervical immunoglobulin levels in women using the copper IUD[140]. IgG, IgA and IgM levels were raised four months following copper IUD insertion. It was suggested that this could be due to a reaction whereby the host recognizes the contraceptive as a foreign body, eliciting an immune response or the increased presence of non-optimal bacteria following insertion[140]. In the past, results emerging from studies assessing the impact of the copper IUD on the vaginal microbiome have been inconsistent. In 2018, Achilles *et al*. reported an increase in BV rates in women using the copper IUD, suggesting that the IUD may increase colonization by BV-associated bacteria in the FGT[110]. Other studies did not establish a link between copper IUD use and changes in the vaginal microbiota[141,142]. As BV has been linked to an increased risk of STI infection, including HIV[143], further investigations of whether and, if so, how the copper IUD impacts the vaginal microbiome are crucial.

As previously discussed, genital inflammation is influenced by many factors, including age and STI status. In this study, concentrations of cytokines also differed between women from different locations. The significant association between copper IUD use and increased levels of IL-6 and IP-10 remained significant after adjusting for possible confounders, namely site, age and infection status with chlamydia, gonorrhoea and HSV-2. This result suggests that the copper IUD may have effects on genital immune mediator profiles regardless of location, age and STI presence.

The increase in both pro-inflammatory, as well as chemotactic cytokines that have been previously linked to increased HIV risk following copper IUD insertion suggests that HIV acquisition risk may be heightened in women using this type of contraceptive. The mechanism behind this may likely be a combination of increased cell target recruitment, promotion of HIV replication and epithelial barrier disruption that are favourable for the establishment of HIV infections. It is important to note that studies assessing the effects of HCs on FGT immunity in the context of HIV risk have used the copper IUD as a non-hormonal control [144]. Given the results of this study, the reliability of this is questionable as immune profiles were significantly changed in the presence of the contraceptive.

DMPA-IM

DMPA-IM is the most commonly used form of HC in sub- Saharan Africa, the area with the highest worldwide HIV-1 prevalence, and epidemiological studies have linked its use to an increased HIV risk[74]. In this study, initiation of DMPA-IM was not associated with any significant changes in immune mediator concentrations. One-month following contraceptive initiation, a slight decrease in SLPI concentrations was observed, however this change was not significant. Three months following DMPA-IM initiation, levels of IFN-α were slightly decreased, but this was not upheld after adjusting for multiple comparisons. Although no substantial changes in immune mediator profiles were detected following DMPA-IM use, the trend was towards an overall reduction. PCA and heat map analysis also showed an association with lower scores on PC1, again indicating immune mediator profiles were either slightly decreased or not changed following DMPA-IM initiation.

SLPI, in particular, stood out as being reduced in DMPA-IM users one-month following initiation. SLPI is a major antimicrobial peptide that contributes to mucosal immunity in the FGT. This small protein has anti-inflammatory properties and is present in saliva, breast milk, genital tract secretions as well as semen. Importantly, at physiological concentrations, it is thought to possess potent anti-HIV-1 activity [145–147]. For example, high levels of SLPI in HIV-infected mothers have been associated with reduced HIV transmission during childbirth[148]. DMPA-IM use has previously been associated with decreased expression of SLPI in endometrial tissue[149] and it is thought that other conditions associated with an increased HIV risk, such as HSV-2 infection and BV, may also downregulate SLPI expression[150,151]. In addition, a recent study by Morrison, *et al.,* established a link between lower SLPI concentrations and HIV seroconversion[18]. Importantly, SLPI is primarily active against R5-tropic viruses, which are responsible for sexual HIV transmission[23,146]. There is a lack of *in vivo* data for mechanisms underlying the antiviral potential of SLPI, however, it has been suggested that its ability to bind cellular cofactors supporting HIV infection of macrophages has inhibitory effects on the virus[152]. Although the picture is incomplete, the role of SLPI as a mediator of innate immunity and its anti-inflammatory potential suggests that a loss of SLPI might contribute to an increased risk of HIV acquisition. Interestingly, DMPA-IM use has also been linked to significant decreases in IFN-α levels due to reduced production of the immune mediator by dendritic cells[153]. In this study, concentrations of IFN-α were decreased three-months following DMPA-IM initiation, although this did not remain significant after adjusting for multiple comparisons. Following SIV infection in rhesus macaques, IFN-α producing

dendritic cells accumulate rapidly beneath the genital mucosa[135]. While the mechanism behind this with regards to DMPA-IM use remains to be explored, suppression of IFN-α production may affect the immune system's ability to control infection and thus benefit the virus[153].

While some animal studies have demonstrated the ability of MPA to suppress immune responses, both systemically and in the FGT[103,154,155], few studies examining this relationship have been conducted in humans. MPA use has, however, been linked to a decrease in the production of several immune mediators, including IFN-α, IL-2, IL-4, IL-6, IL-12, TNF-α and MIP-1α, by peripheral blood cells and activated T cells[153]. In the context of FGT immunity in humans, Ngcapu, *et al*. have previously reported on a decrease of several pro-inflammatory as well as chemotactic cytokines following DMPA-IM use[95]. Although these associations potentially suggest that MPA can interfere with chemotaxis of potential HIV target cells, this must be explored further. Contrary to this down-regulation of immune mediators, some studies have reported on the inflammatory potential of DMPA-IM. Specifically, DMPA-IM use has been associated with increased levels of pro-inflammatory cytokines in vaginal fluids, including IL-6, RANTES, MIP-1α, MIP-1β and IP-10[101]. MPA was also shown to increase IL-6 levels in a human vaginal cell line[156].

Importantly, suppression of the immune responses at the female genital mucosa may influence susceptibility to other infections that in turn may increase HIV infection risk. Studies in mice have shown that HSV-2 risk increases by up to 2-fold when treated with DMPA-IM[108]. Protection against STIs, most importantly HIV, requires a delicate balance of sufficient immune activity, while simultaneously regulating inflammatory conditions at the mucosal point of entry. To date, the effects of DMPA-IM on genial inflammation remain unclear. In the future, it will be important to explore the relationship between serum and local progestin concentrations following the use of MPA-based injectables and their effect on immune mediator profiles. The effects of MPA likely differ greatly between individuals as the local progestin concentrations depend on an array of factors, including the administered dosage, the route of administration as well as the drug metabolism of the individual[73].

LNG implant

No changes in immune mediator concentrations were evident one-month post LNG insertion, however at three months, concentrations of TNF-α, IP-10, MIP-3α and SLPI were significantly raised relative to baseline. This resulted in an overall raised immune mediator profile three months following LNG implant insertion. PCA indicated a shift towards higher concentrations of immune mediators positively loaded on PC1 three months following LNG implant insertion,

further substantiating this observation. Additionally, a higher proportion of women using LNG implant was associated with elevated cytokine levels following unsupervised hierarchical clustering in month three compared to month one.

Tissue-specific and systemic effects in response to LNG implant use are poorly characterized, with no studies assessing changes in immune mediator concentrations. Initial interactions between HIV and mucosal immunity determine the outcome of the exposure in the context of sexual transmission. In order to establish an infection, the virus must cross the mucosal barrier. Physical disruptions in the epithelium can create gaps known as paracellular passages, through which the virus can reach the submucosa[157]. High levels of pro-inflammatory cytokines, including TNF-α, which was raised in LNG implant users in this study, can lead to reorganization of the actin-cytoskeleton and destabilization of tight junctions, leaving the mucosa vulnerable to infection[157]. A recent study showed that LNG reduced genital expression of the desmosomal cadherin desmoglein-1α (DSG1α) in mouse models[96]. Desmosomes are cellular structures specialized for cell-to-cell adhesion and therefore crucial to the integrity of the mucosal barrier[158]. Downregulation of these structures resulted in enhanced access of inflammatory cells to the genital mucosa by increasing mucosal epithelial permeability and thus led to an increased susceptibility to infections[96]. A second possible mechanism for establishment of infection is transcytosis. During this process, viral particles bind to molecules on the epithelial cell surface, are then internalized to form vesicles and can be transported into the intracellular environment. The presence of pro-inflammatory cytokines, including TNF-α, can trigger the release of virion particles from vesicular compartments at the intracellular space, allowing them to infect target cells at the lamina propria[157]. Although it is well described that TNF-α levels are raised during HIV exposure[159,160], the presence of high levels of this cytokine could affect the mucosal barrier integrity, leaving the FGT vulnerable to infections including HIV. This vulnerability may be further enhanced by the presence of high levels of chemotactic cytokines, such as IP-10. As previously discussed, elevated levels of IP-10 are thought to increase target cell recruitment, providing more hosts to HIV and thus increasing the risk of infection.

It is important to note that LNG implant use, relative to DMPA-IM use, remained significantly associated with higher levels of genital IP-10 and SLPI three months following contraceptive initiation after controlling for confounders (site, age and infection status with chlamydia, gonorrhoea and HSV-2). Although SLPI has been found to be protective against HIV infection at high levels in some studies[145–147], a recent study reported that higher cervicovaginal lavage

concentrations of SLPI were associated with HIV seroconversion[161] and another identified an association between lower SLPI concentrations and HIV seroconversion risk in a larger populations[162]. However, it has been suggested that higher levels of HIV-1 RNA in the FGT may induce epithelial cell expression of SLPI[161]. This means that the association between higher SLPI levels pre-infection and increased HIV acquisition risk could be due to HIV-1-induced expression of SLPI by epithelial cells in women that have experienced repeated HIV exposure[161]. However, this idea needs to be explored further.

The available data on the effects of LNG implant use on genital cytokine profiles are sparse, however, a recent meta-analysis suggested no link between LNG implant use and an increased risk of HIV acquisition[74]. Contrasting this, the results of this study indicate that LNG use may affect genital immune mediator levels, warranting further research.

Limitations

BV data was not available for this study and women were only tested for *N. gonorrhoeae* and *C. trachomatis* at baseline. Further testing for STIs and BV at all time-points will, however, be conducted on stored samples in the future. Additionally, this study only evaluated changes in immune mediators up to three months and it is therefore unclear whether the inflammatory responses observed would subside over a longer period of time.

Conclusion

The debate surrounding the effects of HCs on genital immune mediator profiles, and by extension HIV risk, is ongoing. This study did not establish a link between DMPA-IM use and significant changes in the concentrations of the genital immune mediators that were measured. Although not significant, the trend was towards a decrease in overall immune mediator profiles three months following DMPA-IM initiation. While results assessing the effects of HCs on genital cytokine profiles are inconsistent, this is particularly true for the injectable DMPA-IM. Some studies have reported an increase in concentrations of important genital immune mediators following DMPA-IM use[131] and others have suggested the opposite[95]. In the future, the immunosuppressive potential of MPA should be assessed following the use of Sayana Press, a lower dose formula than DMPA-IM that is administered subcutaneously[163].

This study additionally showed that the copper IUD and LNG implant were associated with a rapid increase of immune mediator levels. Interestingly, IP-10 has been identified as one of the most critical cytokines elevated in women that later became HIV-infected[4] and this chemokine was found to be elevated in both copper IUD and LNG implant users in this study. Its

chemotactic effects on HIV target cells in the genital mucosa could play an important role in the context of HIV acquisition risk. Recruitment of potential target cells could be further enhanced by the presence of high levels of other chemotactic cytokines, such as IL-8, MIP-1α and MIP-1β, as seen in copper IUD users, and MIP-3α, as seen in LNG implant users. In copper IUD users, the increase in chemokine levels was accompanied by significantly increased concentrations of the pro-inflammatory cytokine IL-6. As previously discussed, IL-6 is thought to play an important role in HIV risk by promoting mucosal inflammation and HIV expression[60].

Overall, this study has shown that both non-hormonal contraception in the form of the copper IUD and HCs, in this case the LNG implant, have the potential to alter immune mediator profiles in the FGT. HIV susceptibility in the FGT is known to be enhanced by several downstream effects of raised cytokine levels, including increased target cell recruitment and impaired mucosal barrier integrity. Furthermore, genital inflammation is thought to impair the effectiveness of HIV prevention methods[19]. More studies assessing the effects of contraceptive methods on genital inflammation and its downstream effects on HIV acquisition risk are needed to complete the picture.

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Appendix

Luminex assay principle

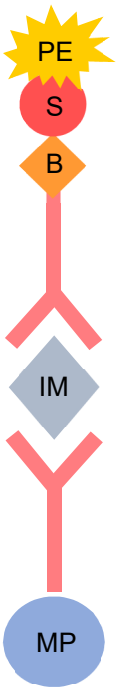

Figure S1. Luminex assay principle. Colour-coded magnetic microparticles (MP) that are pre-coated with immune mediator (IM)-specific antibodies are supplied by the manufacturer. Together with standards or samples and the specific antibodies, these microparticles are added to wells. The specific antibody binds to the analyte of interest. Substances that do not bind to the antibodies are washed off and biotinylated antibodies specific to the analytes of interest are added. A second wash removes unbound biotinylated (B) antibodies. Phycoerythrin (PE)-conjugated streptavidin (S) is added and binds to the biotinylated detection antibodies. Unbound Streptavidin-PE is removed with a last wash. The microparticles are then resuspended in buffer to be read with a Luminex Analyzer. The microparticles are held in a monolayer with the use of a magnet and the beads are illuminated by 2 Light Emitting Diodes (LEDs). One laser classifies the bead and determines the analyte that is being detected, while the other measures the magnitude of the PE-derived signal. The intensity is directly proportional with the amount of analyte that is bound. This information is recorded using a CCD camera. [(According to R&D Manual), R&D Systems, Inc., USA]. MP: microparticle, IM: immune mediator, B: biotin, S: streptavidin, PE: phycoerythrin.

Standard curves

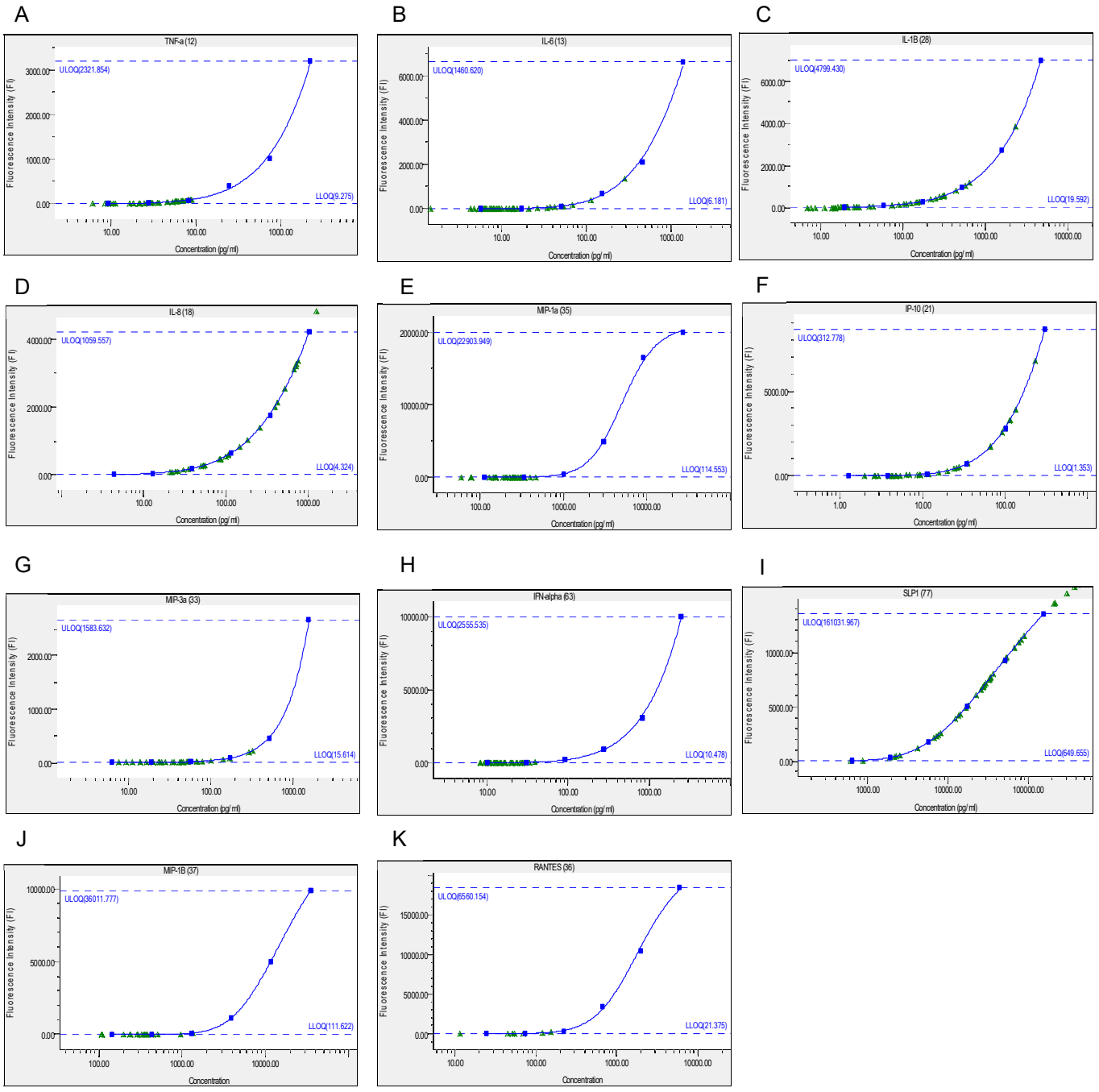

Figure S2. **Examples of Standard curves generated during Luminex.** Genital immune mediator concentrations (pg/mL) in lateral vaginal wall swabs were measured using Luminex. Standards are displayed as blue rectangles, samples are represented by green triangles. Abbreviations: TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted.

Line graphs of genital immune mediator concentrations over time

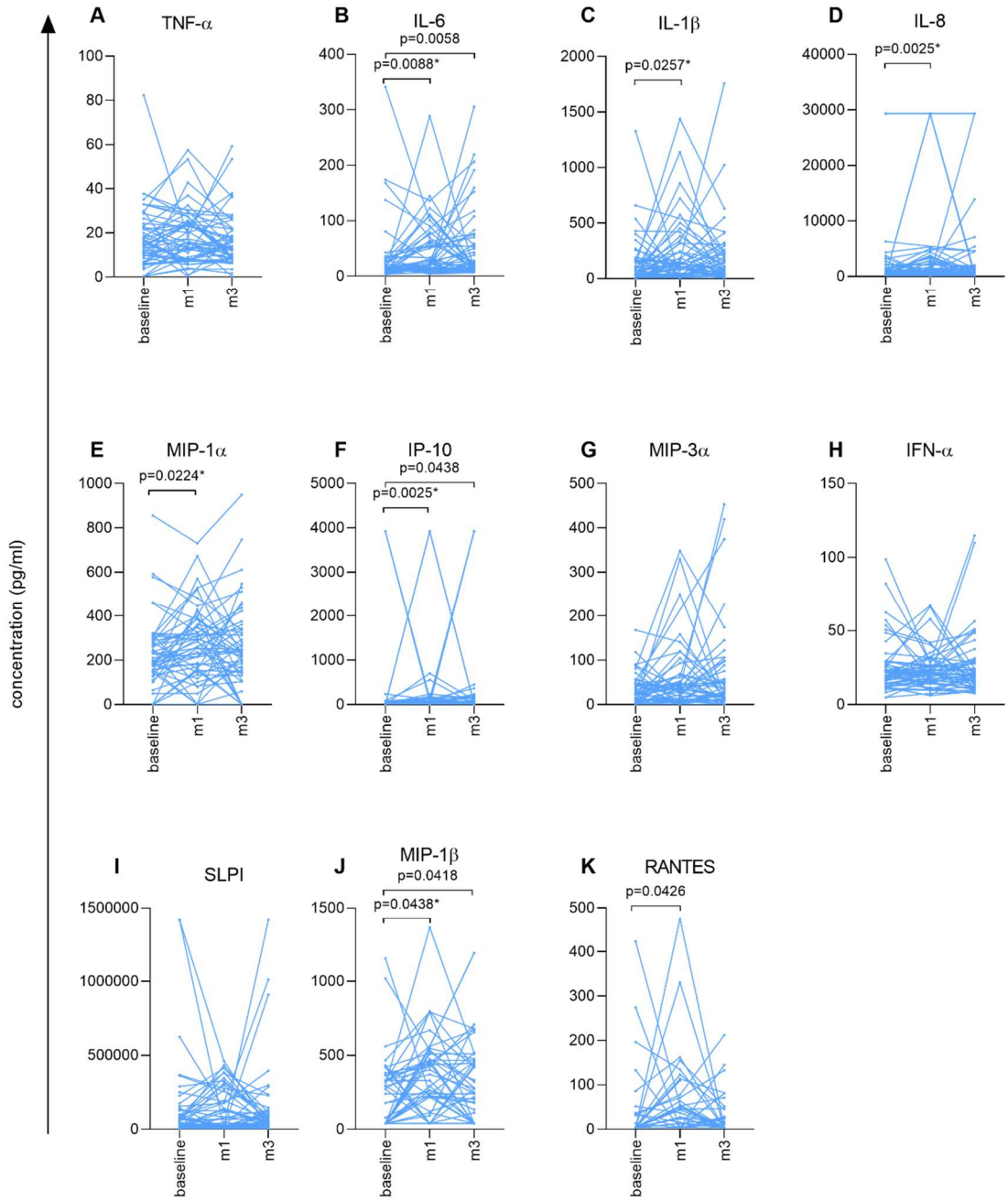

Figure S3 (A-K). Line graphs of genital immune mediator concentrations (pg/mL) at baseline, one month following insertion of Cu-IUD (m1) and three months following insertion of Cu-IUD (m3) (sample size, n=55). Genital immune mediator concentrations in lateral vaginal wall swabs were measured using Luminex. Wilcoxon signed rank test was used for comparisons and p-values were adjusted for multiple comparisons using a false discovery rate step-down procedure. Abbreviations: Cu-IUD, Copper intrauterine device; TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted. * Indicates $p < 0.05$ after adjusting for multiple comparisons.

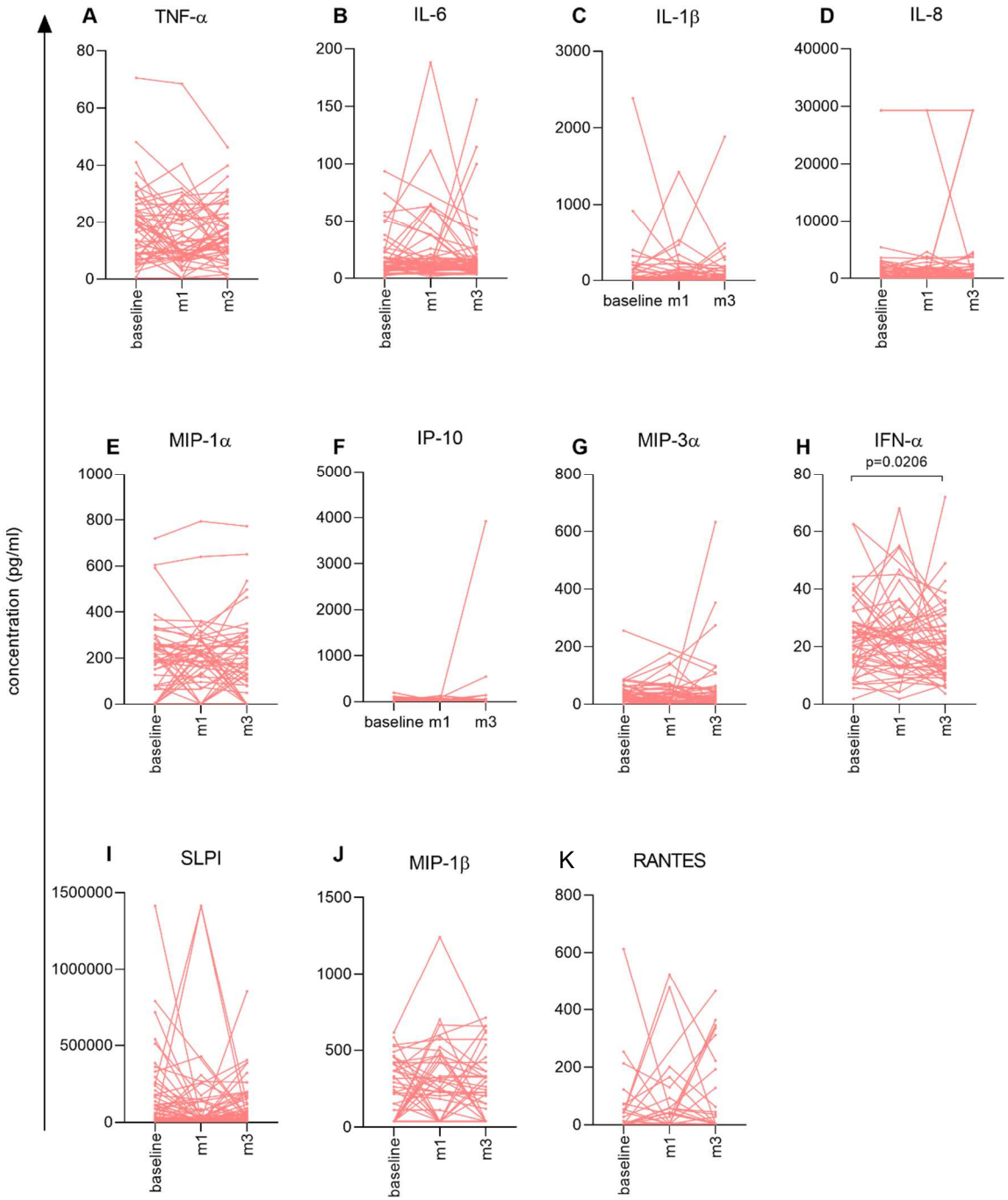

Figure S4 (A-K). Line graphs of genital immune mediator concentrations (pg/mL) at baseline, one month following initiation of DMPA-IM (m1) and three months following initiation of DMPA-IM (m3) (sample size, n=53). Genital immune mediator concentrations in lateral vaginal wall swabs were measured using Luminex. Wilcoxon signed rank test was used for comparisons and p-values were adjusted for multiple comparisons using a false discovery rate step-down procedure. Abbreviations: DMPA-IM, intramuscular depomedroxyprogesterone acetate; TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted. * Indicates $p < 0.05$ after adjusting for multiple comparisons.

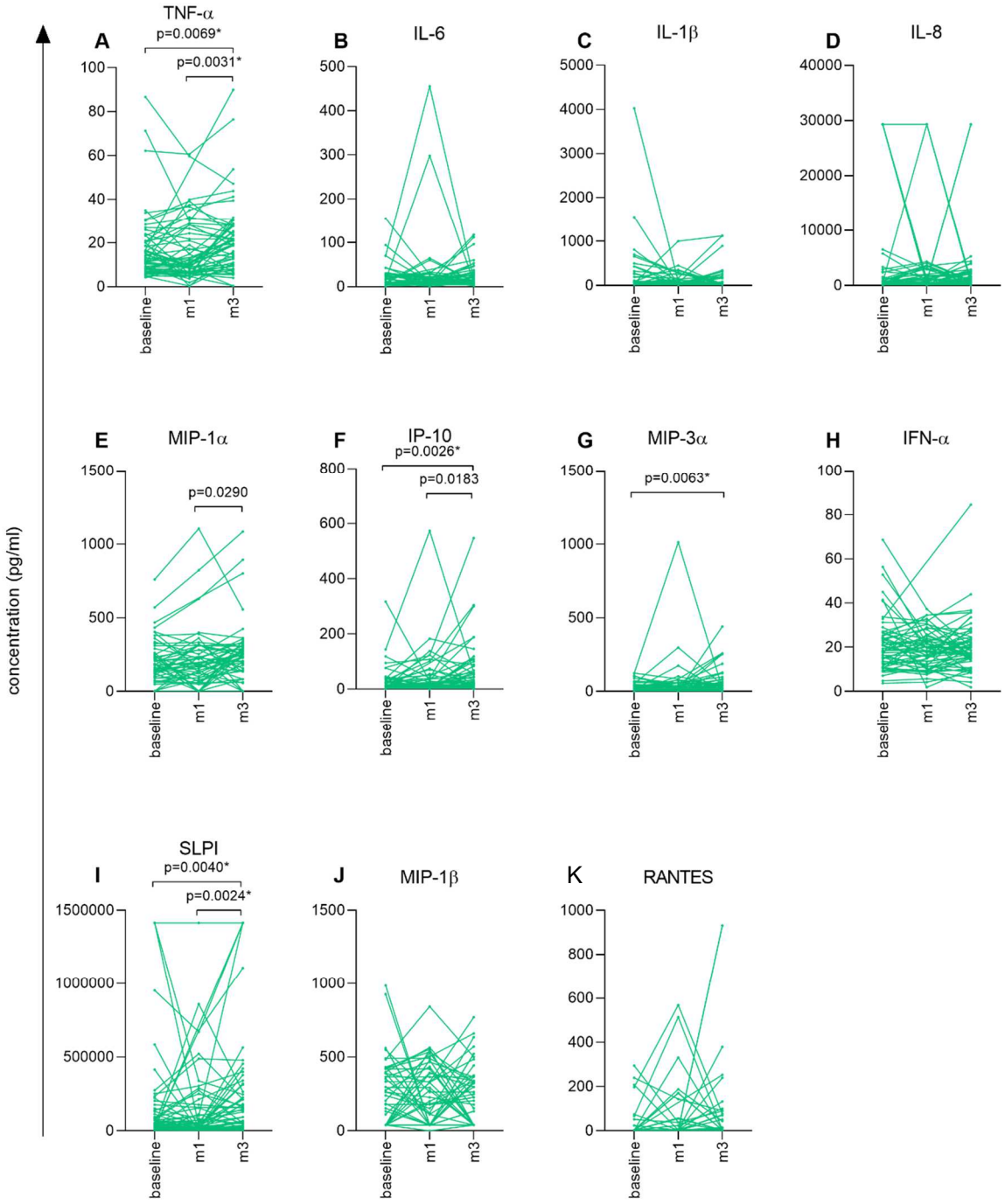

Figure S5 (A-K). Line graphs of genital immune mediator concentrations (pg/mL) at baseline, one month following insertion of LNG Implant (m1) and three months following insertion of LNG Implant (m3) (sample size, n=59). Genital immune mediator concentrations in lateral vaginal wall swabs were measured using Luminex. Wilcoxon signed rank test was used for comparisons and p-values were adjusted for multiple comparisons using a false discovery rate step-down procedure. Abbreviations: LNG, levonorgestrel; TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted. * Indicates $p < 0.05$ after adjusting for multiple comparisons.

Log_{10}-transformed fold change analysis of immune mediator data

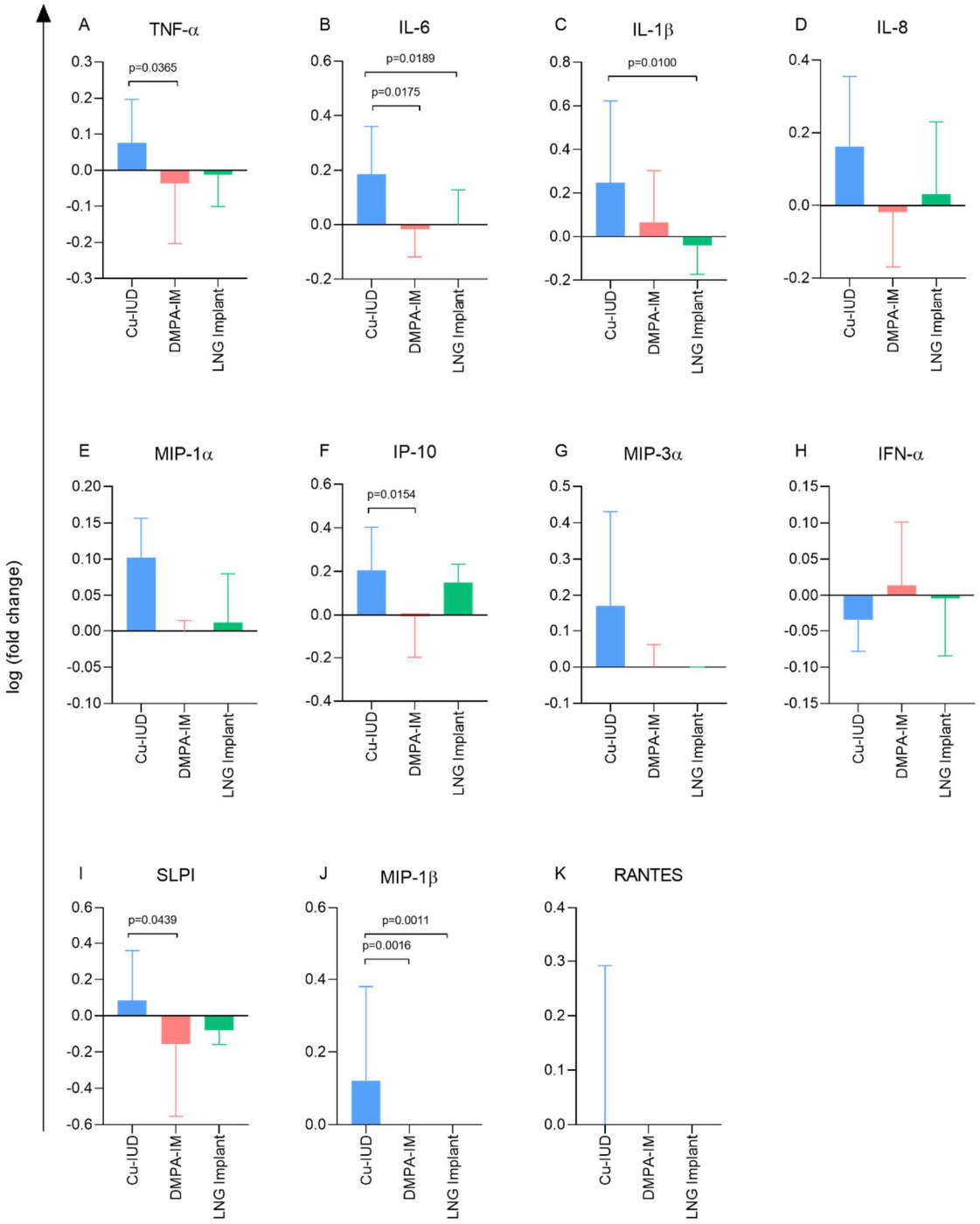

Figure S6 (A-K). Log_{10}-transformed fold changes of genital immune mediator concentrations one month following initiation of contraceptive method in women using copper IUD (n=52), DMPA-IM (n=50) and LNG Implant (n=56). Genital immune mediator concentrations in lateral vaginal wall swabs were measured using Luminex. Mann-Whitney U test was used for comparisons and p-values were adjusted for multiple comparisons using a false discovery rate step-down procedure. Abbreviations: Cu-IUD, Copper intrauterine device; DMPA-IM, intramuscular depo-medroxyprogesterone acetate; LNG, levonorgestrel; TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted.

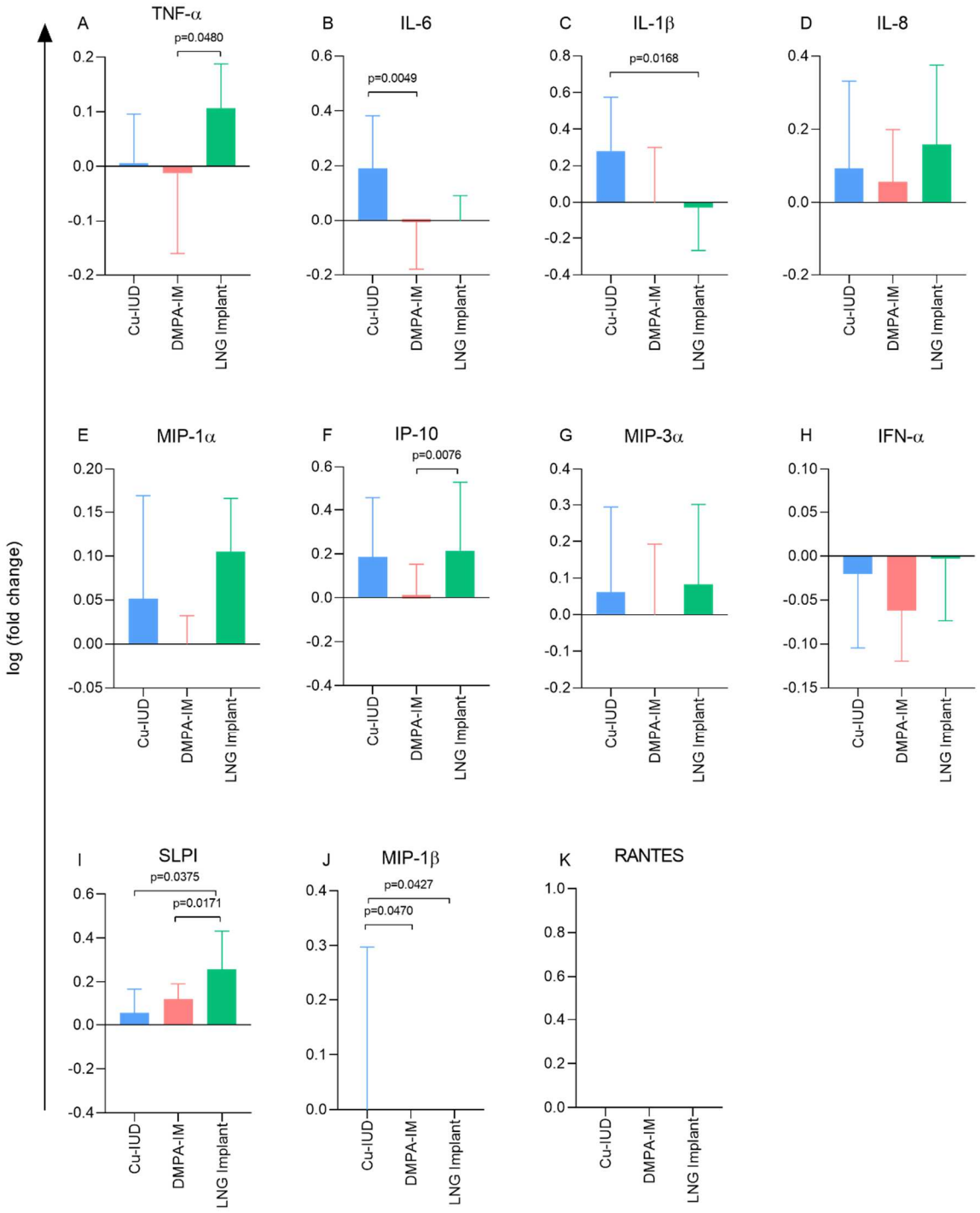

Figure S7 (A-K). Log_{10}-transformed fold changes in genital immune mediator concentrations three months following initiation of contraceptive method in women using copper IUD (n=55), DMPA-IM (n=53) and LNG Implant (n=59). Genital immune mediator concentrations in lateral vaginal wall swabs were measured using Luminex. Mann-Whitney U test was used for comparisons and p-values were adjusted for multiple comparisons using a false discovery rate step-down procedure. Abbreviations: Cu-IUD, Copper intrauterine device; DMPA-IM, intramuscular depo-medroxyprogesterone acetate; LNG, levonorgestrel; TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted.

Baseline immune mediator profiles of women positive for *Chlamydia trachomatis*

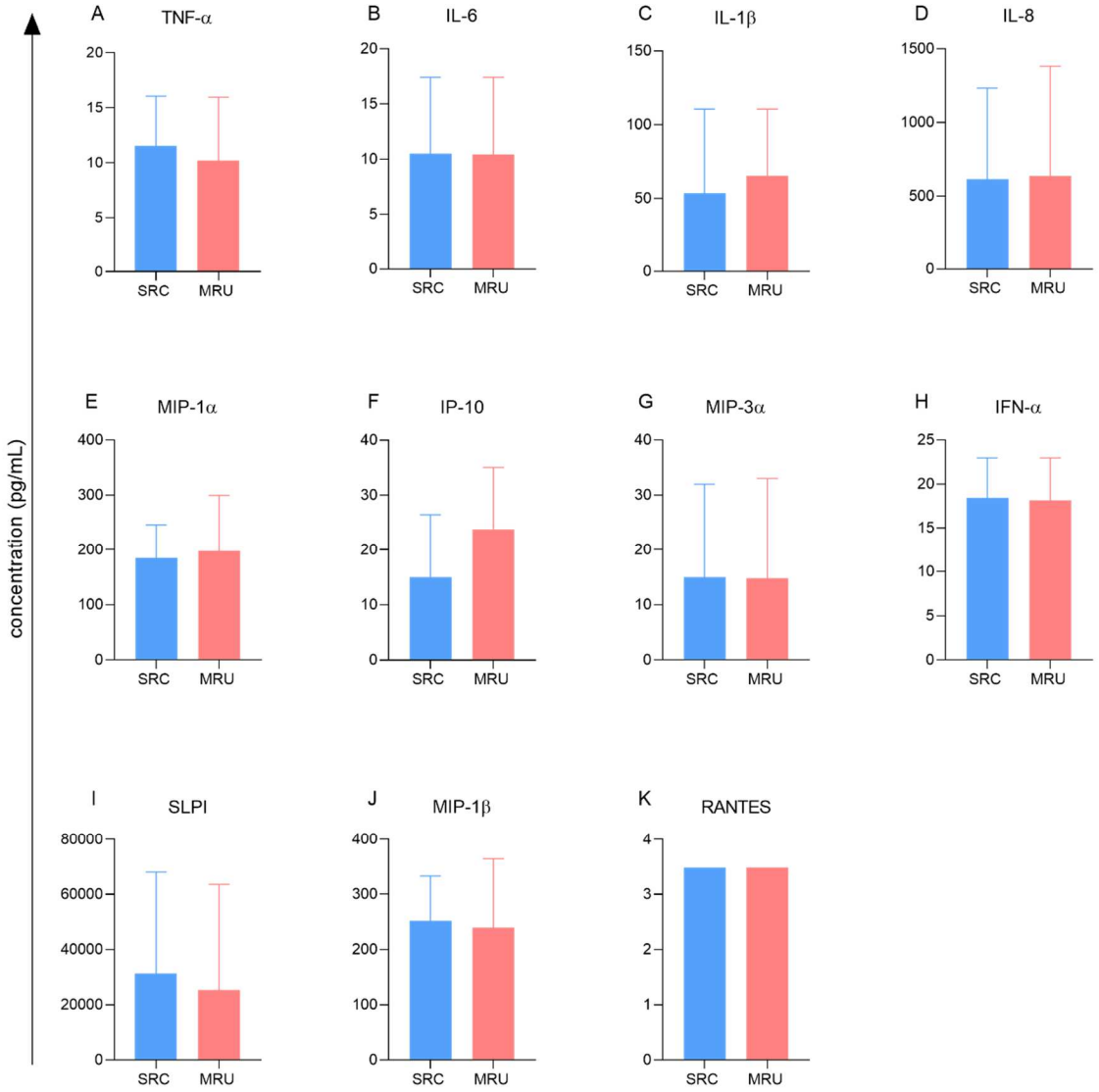

Figure S8 (A-K). Baseline genital immune mediator profiles of women positive for *Chlmaydia trachomatis*, stratified by site. Genital immune mediator concentrations in lateral vaginal wall swabs were measured using Luminex. Women tested positive for Chlamydia were grouped by site; Setshaba Research Centre (blue; n=42) and MatCH Research Unit (pink; n=31). Mann-Whitney U test was used for comparisons. Abbreviations: SRC, Setshaba Research Centre (Pretoria); MRU, MatCH Research Unit (Durban); TNF-α, tumour necrosis factor-alpha; IL, interleukin; MIP, macrophage inflammatory protein; IP-10, interferon-γ inducible protein-10; IFN-α, Interferon-alpha, SLPI, secretory leukocyte protease inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted.

Average age of women from SRU and MRU

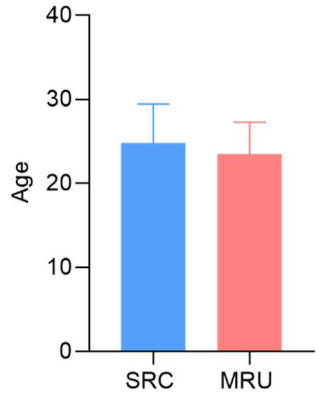

Figure S9. Average age of women from Setshaba Research Centre (blue, n=54) and MatCH Research Unit (pink, n=113). Mann-Whitney U test was used to compare groups. Abbreviations: SRC, Setshaba Research Centre (Pretoria); MRU, MatCH Research Unit (Durban).